Quality and Reliability of Prenatal Cytogenetics

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Quality and Reliability of Prenatal Cytogenetics

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Miles of the unknown

The road ahead is empty
It's paved with miles of the unknown
Whatever seems to be your destination
Take life the way it comes
Take life the way it is

Cito to city

Contents

Chapter 1

General introduction

1.1 Brief history of prenatal diagnosis

1.1.1 Amniocentesis

In the early fifties, prenatal investigation of amniotic fluid started with the evaluation of Rhesus sensitization (Bevis, 1950, 1952). It was followed by the discovery that fetal gender could be determined by the presence or absence of a sex chromatin body in the nuclei of cells in the amniotic fluid (Fuchs and Riis, 1956).
In 1966, Steele and Breg demonstrated the possibility of culturing and karyotyping viable amniotic fluid cells, mainly of epithelial origin. Further progress was made with the refinement of the technique and timing of amniocentesis (Thiede et al., 1966; Jacobson and Barter, 1967), and the first prenatal diagnosis of Down syndrome (Valenti et al., 1968).
Other important developments, in the same period, were the use of amniocentesis for the prenatal detection of biochemical abnormalities (Nadler and Gerbie, 1968), the development of ultramicrochemical techniques for rapid prenatal biochemical diagnosis (Galjaard et al., 1972, 1977, 1980; Niermeijer et al., 1975), and the finding of an association between a raised concentration of alpha-fetoprotein in amniotic fluid and an open neural tube defect of the fetus (Brock and Sutcliffe, 1972). Cytogenetic investigations were improved by the discovery of the G-banding technique (Seabright, 1971).
For almost fifteen years amniocentesis was the only procedure for prenatal investigations. At the end of the eighties, more than a quarter of a million amniocenteses had been performed and the number to date is probably in the millions. Nowadays, the safety of second trimester amniocentesis together with the reliability, accuracy and efficiency is well recognized; it is generally considered as the "gold standard".
Amniocentesis is usually performed around the 16th week of pregnancy with a cytogenetic result available after 10 - 18 days. Second trimester termination of pregnancy is not free from medical complications and causes psychological stress (Leschot et al., 1982; Thomassen Brepols, 1985). For this reason, developments in prenatal diagnosis have led to various new or modified techniques during the past decades, among others, amniocentesis prior to 15 weeks of gestation (early amniocentesis). It has been established that early amniocentesis is not a suitable alternative to regular amniocentesis because of a higher risk of pregnancy loss. Today, chorionic villus sampling has become the test for first-trimester prenatal diagnosis (Cederholm and Axelsson, 1997; Winsor et al., 1999; Johnson et al., 1999).

1.1.2 Chorionic villi

Although the first techniques for 'transcervical' chorionic villus sampling (CVS) were described in 1958 by Acosta-Sisen, and for 'transabdominal' CVS in 1966 by Alvarez et al., the first concept of CVS for fetal diagnosis was published in 1968 by Mohr.

He used hysteroscopy and was remarkably successful in obtaining villi from patients prior to elective abortion. Complications as damage to the amniotic sac, maternal bleeding, the absence of real-time ultrasound and the low success rate of chorionic villus cell culture (Hahnemann, 1974; Kullander & Sandahl, 1973), together with the rapid acceptance of amniocentesis delayed any major research in western countries. A revival occurred after a publishing in China that CVS was successfully used as a method of fetal sex prediction (Anshan Iron and Steel Company, 1975). Russian scientists subsequently demonstrated the feasibility of CVS in early prenatal diagnosis of inborn errors of metabolism (Kazy et al., 1982).

The breakthrough of CVS came with the demonstration that chorionic villi could be used for cytogenetic diagnosis without the need of cell culture (Simoni et al., 1983). From that time on, CVS and karyotyping of uncultured chorionic villi became an attractive alternative to amniocentesis. The advantages were obvious; the procedure was carried out early in pregnancy, the results were rapidly available, providing a diagnosis at 11 - 13 weeks allowing termination of pregnancy by suction curettage as an outpatient procedure. The first prenatal diagnosis in The Netherlands in a non-experimental CVS was performed in June 1983 (Galjaard, 1985; Sachs et al., 1983, 1985; Jahoda et al., 1984).

After the first publication of Brambati and Simoni in 1983, trials with CVS were started in many centres in order to gain experience with the sampling and the laboratory investigations. Chorionic villus sampling became popular and developed rapidly into a widely used prenatal diagnostic procedure not only for chromosomal disorders, but also for metabolic disorders (for early reviews see Galjaard, 1985; Kleijer et al., 1986). DNA analysis was found to be possible for haemoglobinopathies (Old et al., 1986) and Duchenne muscular dystrophy (Bakker et al., 1985)(see for early reviews Fraccaro et al., 1985).
After almost 10 years of increasing numbers of CVS with concomitant decreasing numbers of amniocenteses there was a turning point due to unsolved problems associated with CVS. This trend is also reflected in the results of our own centre as shown in Figure 1.

A number of questions can be raised as to why first-trimester CVS had not replaced second-trimester amniocentesis.

1) Is there an excess of any sampling related risk for the woman and her fetus in comparison to amniocentesis concerning:
    - Pregnancy loss
    - Vascular disruptive syndromes in the fetus
    - Blood transfusion between mother and fetus
2) Are there more laboratory related problems in comparison to amniotic fluid concerning:
    - Laboratory failures
    - Quality of chromosome preparations
    - Discrepancy between prenatal diagnosis and fetal status

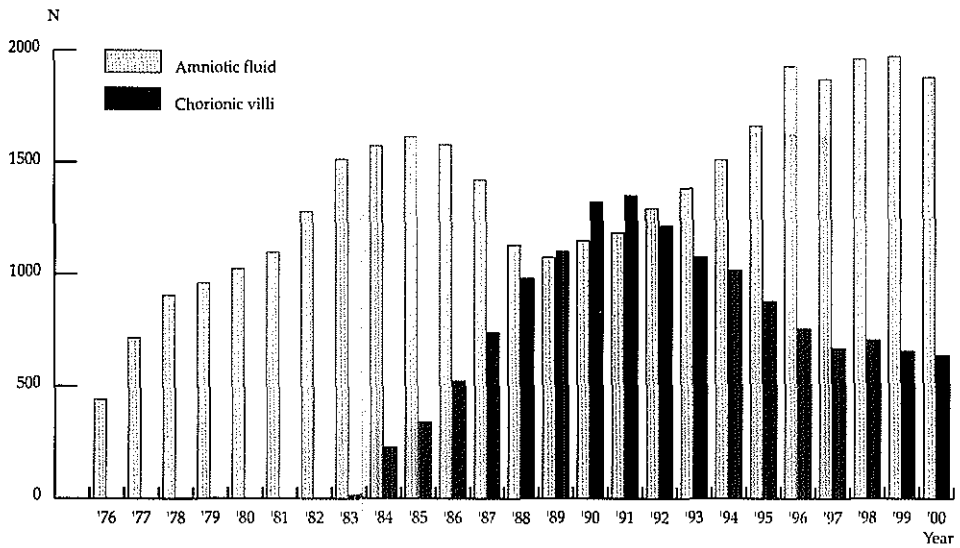

Figure 1 - Number of amniotic fluid and chorionic villi during the years 1976 - 2000

1.2 Sampling related risks

1.2.1 Pregnancy loss

The procedure related risks of pregnancy loss following CVS and amniocentesis have been studied extensively world-wide but were difficult to assess due to differences in methodology, and the gestational age at the time of the procedure.
For amniocentesis the risk has been estimated up to 1% (fetal loss before 28 weeks of gestation)(NICHD, 1976; MRC working party, 1978; Golbus et al., 1979; Sachs et al., 1982,; Canadian collaborative CVS-Amniocentesis clinical trial group, 1989; Brandenburg, 1992; Antsaklis, 2000). Transplacental amniocentesis, blood stained amniotic fluid, a history of previous spontaneous or induced abortions, and bleeding in the current pregnancy turned out to increase the risk of fetal loss (Tabor et al., 1986; Brandenburg, 1992; Antsaklis, 2000).
For CVS, the situation is more difficult to evaluate since the sampling takes place in the period of a relatively high rate of spontaneous abortion and two different modes of sampling are used (transcervical (TC) and transabdominal (TA) sampling). Differences between TC- and TA- CVS associated risks could not be demonstrated in two studies (Brambati et al., 1991; Jackson et al., 1992), but in two other studies the

risk of TC-CVS was found to be significantly higher than that of TA-CVS, whilst the risk of TA-CVS was found to be equal to that of amniocentesis (Philip et al., 1991; Smidt-Jensen et al., 1992).
It is generally accepted that the risk of CVS associated pregnancy loss shows an inverse correlation with the skill of the operator. The "learning curve" effect for CVS takes 50-150 procedures, depending on the operator to achieve a minimal fetal loss rate (Leschot et al., 1989; Rhoads et al., 1989; Jackson et al., 1992; Wijnberger et al., 2000). According to quality standards issued by the Dutch Society of Obstetrics and Gynaecology (NVOG), qualified gynaecologists have to perform a minimum of 30 supervised procedures before operating on their own patients. The WHO/PAHO (1999) has stated that the risk of pregnancy loss is equal after amniocentesis and TA-CVS in experienced hands.

1.2.2 *Vascular disruptive syndromes*

After the first report of Firth et al. (1991) on limb reduction after CVS, this matter has been studied intensively without clear conclusions. In some studies an association was found between vascular disruptive syndromes and CVS (Mastroiacovo and Cavalcanti, 1991; Burton et al., 1992, 1995; Firth et al., 1994, 1997; Olney et al., 1995), while in other studies no association was found (Schloo et al., 1992; Froster and Jackson, 1996; WHO/PAHO, 1999). In all studies it was established that in the case of limb reduction, the gestational age at sampling ranged from 8 to 11 weeks. The general conclusion has been that CVS should be postponed to late first trimester (11 - 12 weeks)(NICHD, 1993).

1.2.3 *Blood transfusion between mother and fetus*

Theoretically, the only sampling related risk left is transfusion of blood between mother and fetus. Feto-maternal-transfusion (FMT) can occur spontaneously early in pregnancy, but is also known to take place in association with CVS (Los et al., 1989; Smidt-Jensen et al., 1993; Jansen et al., 1997). Since large FMT-volumes have been established without apparent effect on the pregnancy outcome it has been speculated that FMT might be followed by materno-fetal-transfusions (MFT) as a compensatory exchange transfusion (Los et al., 1996). MFT might introduce vaso-active substances into the fetal circulation and cause a vascular accident in the fetus (Quintero et al., 1992; Los et al., 1999). However, to our knowledge there is only one case of fetal demise clearly documented to be caused by FMT (Los et al., 1993) and therefore, FMT remains a theoretical risk.

1.3. Laboratory related problems

1.3.1 *Laboratory failure*

One of the potential problems is a laboratory failure, consisting of the failure of cell growth, infection, or harvesting failures, resulting in insufficient numbers of metaphases, or even no metaphases at all necessary for chromosome analysis.
Fortunately, the frequency of laboratory failures is very low. For amniotic fluid and chorionic villi the laboratory failure rate is 0.1% - 0.4% and 0.3% - 2.0%, respectively. (Ledbetter et al., 1990, 1992; Lippman et al., 1992; Smidt-Jensen et al., 1993; Lam et al., 1998; Waters et al., 1999). Our own figures are just at the upper limit for amniotic fluid (0.40%) and in the lower region of the reported range for chorionic villi (0.54%) (Los et al., in press).

1.3.2 *Quality of chromosome preparations*

Another potential laboratory related problem is poor quality of chromosome morphology. The chromosome quality is based on the subjective judgement of the technicians and the cytogeneticist. An important parameter characterizing the quality of the metaphases is the banding resolution; the ISCN (International System for Human Cytogenetic Nomenclature) is adhered in order to define the total number of bands in the metaphases (ISCN, 1985, 1995; ACC, 1988).
It is well known that the quality of cytogenetic preparations depends on the type of cells studied and that the quality varies from preparation to preparation. The 400 band resolution level should be the aim for prenatal specimens. However, it is not always possible to achieve this.
The number of bands observed in metaphases of short-term cultures of chorionic villi cells (STC-villi)(trophoblast cells) is between 200 and 400, which is in fact below the usual preferred standard. However, the combined use of STC-villi and long-term culture (LTC-villi)(cultured cells of the mesenchymel villus core) can improve the quality of chromosome analysis and results in an acceptably high quality compared with that of amniotic fluid cells. Figure 2A + 2B show karyotypes of a fairly good STC-villi preparation with approximately 300 bands and an average quality karyotype of LTC-villi displaying about 400 bands from the same sample. The number of bands observed in metaphases of cultured amniotic fluid cells meet the required band level. Figure 3 shows an average quality karyotype from amniotic fluid cells also displaying about 400 bands.
It may be argued that the number of bands reflects the accuracy with which small structural chromosomal abnormalities can be determined. The last few years, the accuracy has been enhanced by the use of fluorescence in situ hybridization using DNA probes (FISH) (Klinger et al., 1992; Lebo et al., 1992; Van Opstal, 1998).
FISH has proved to be a useful tool for the detection of minor (familial) chromosome rearrangements, but it cannot yet replace conventional karyotyping.

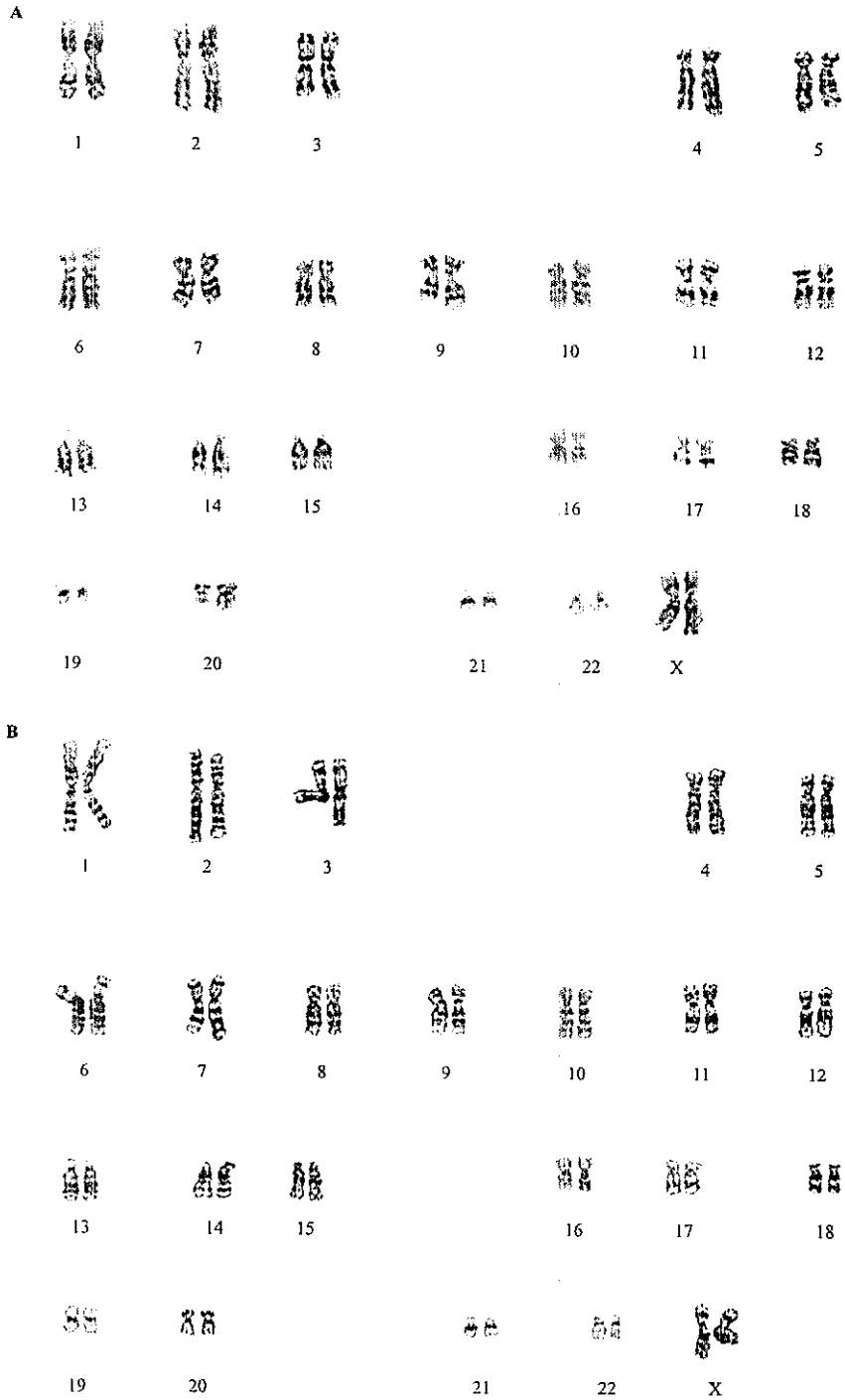

Figure 2 - Karyotype of a fairly good STC-villi preparation (A) and an average LTC-villi preparation (B)

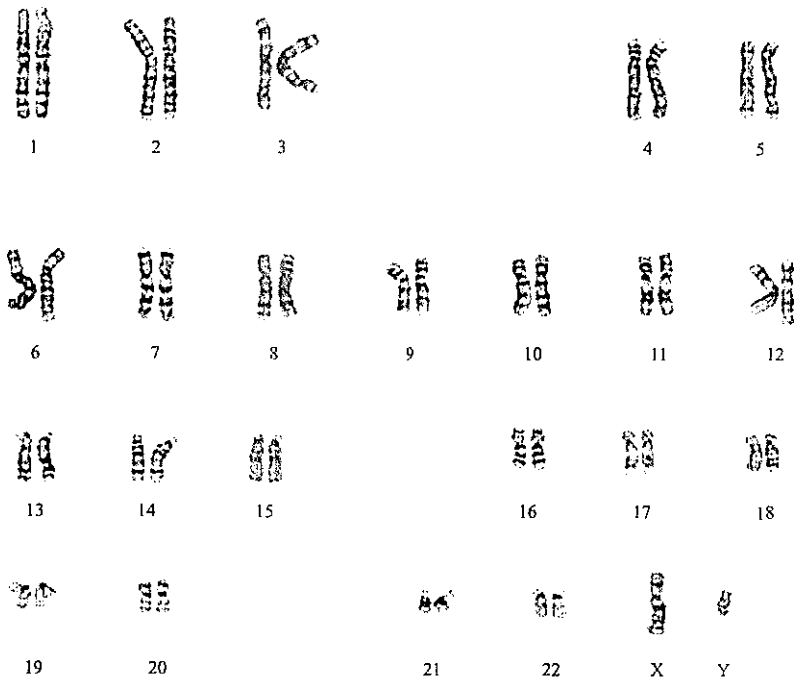

Figure 3 - Karyotype of amniotic fluid cells

1.3.3 *Discrepancy between prenatal diagnosis and fetal status*

Probably the most serious laboratory related problem is discordance between the prenatal cytogenetic diagnosis and the outcome of the pregnancy.
The finding of chromosomal mosaicism in amniotic fluid cells or chorionic villi preparations poses a significant problem in prenatal diagnosis because of the difficulty of determining whether the mosaicism reflects the true chromosomal constitution of the fetus. Mosaicism is caused by an abnormal cell division resulting in the presence of two or more cells with an identical abnormality, among the other normal cells. Because of the diagnostic importance a separate section will be devoted to this topic.

1.4 Chromosomal mosaicism

1.4.1 Amniotic fluid and fetus

In amniotic fluid, two types of mosaicism are known: pseudomosaicism and true mosaicism. If the aberrant cells are confined to a single colony or to one culture dish/flask, it is defined as a pseudomosaicism, whereas a true mosaicism involves the presence of aberrant cells/colonies in two or more independent culture dishes/flasks. Pseudomosaicism is believed to originate in vitro, and hence, to be without clinical consequences. Based on the number of abnormal cells/colonies detected and their distribution within the culture dishes, a classification of 4 types has been made (Boué et al., 1979):

1. Pseudomosaicism type A; one cell or one region in a colony abnormal
2. Pseudomosaicism type B; all the cells of one single colony abnormal
3. Pseudomosaicism type C; multiple colonies within the same culture dish abnormal
4. True mosaicism; two or more abnormal colonies in at least two independent culture dishes

Pseudomosaicism occurs in 0.6 - 1.1% of amniocenteses (Bui et al., 1984; Hsu and Perlis, 1984; Worton and Stern, 1984), with the majority of cases being type A.
Since, these 'in-vitro' events have no clinical significance they are generally not reported to clinicians because of the negligible risk for a true mosaicism (Hsu et al., 1984).
The frequencies of true mosaicism range from 0.1 - 0.3%, with an average rate of cytogenetic confirmation of 70% in fetal tissue (Bui et al., 1984; Hsu and Perlis, 1984; Worton and Stern, 1984).

The method for distinguishing pseudomosaicism from a potentially true mosaicism is increasing the number of metaphases in other dishes than the dish in which the abnormal cells/colonies were present. The investigation of an insufficient number of cells might erroneously designate a true mosaicism as a pseudomosaicism and lead to a false-negative prenatal cytogenetic diagnosis. In 1992, Hsu et al. developed two different levels of work-up, with a modification in 1999, for the differentiation between pseudomosaicism and true mosaicism (Table 1). Extensive and moderate additional work-up mean the analysis of 24 and 12 cell colonies, respectively, the initial dish with the abnormal cells/colonies not included. According to Hook (1977) this can rule out a 12% and 23% level of mosaicism at a 95% confidence level, respectively.
The use of these guidelines results in an accurate and reliable diagnosis of pseudomosaicism or true mosaicism in cultured amniotic fluid cells.

Table 1 - Guidelines for work-up for the differentiation between pseudomosaicism and mosaicism (Hsu et al., 1992, 1999)

A. Indications for extensive work-up (24 colonies exclusive the "affected" dish)

* Autosomal trisomy involving chromosomes 2, 5, 8, 9, 12, 13, 14, 15, 18, 20, 21, 22 (SCo, MCo)
* Unbalanced structural rearrangement (MCo)
* Marker chromosome (MCo)

B. Indications for moderate work-up (12 colonies exclusive the "affected" dish)

* Autosomal trisomy involving a chromosome 1, 3, 4, 6, 7, 10, 11, 16, 17, 19 (SCo, MCo)
* Unbalanced structural rearrangement (SCo)
* Marker chromosome (SCo)
* Extra sex chromosome (SCo, MCo)
* 45,X (SCo, MCo)
* Balanced structural rearrangement (MCo)

C. No additional work-up (routine analysis of 16-20 colonies from ≥2 dishes)

* Balanced structural rearrangement (SCo)
* Break at centromere with loss of one arm (SCo)
* All single cell abnormalities

SCo = single colony/single dish; MCo = multiple colonies/single dish

1.4.2 Chorionic villi and fetus

While acquiring experience with cytogenetic diagnosis in chorionic villi, especially in direct- and STC-villi preparations, it became clear that this did not always reflect the chromosomal constitution of the fetus (Mikkelsen, 1985; Leschot et al., 1987, 1989, 1990; Sachs et al., 1990). During the past decade, it became clear that the reliability of cytogenetic diagnosis could be improved by the combined use of STC- and LTC-villi preparations (Ledbetter et al., 1992; ACC, 1994; Hahnemann and Vejerslev, 1997).

Although fetus and placenta originate from the same zygote, their chromosomal constitution can be different. This is the prevalent problem with CVS as documented by numerous cases of discordant findings in fetus and placenta known as confined placental mosaicism (CPM) (Kalousek and Dill, 1983).
Based on the position of the different cell lines and the knowledge of the fetal karyotype, three general types of discrepancies can occur (Kalousek, 1990).
Table 2 shows the classification of CPM which is used by most centres.

Table 2 - Classification of CPM according to Kalousek et al (1990)

	Type I	Type II	Type III
Direct- and STC-villi	Abnormal/Mosaic	Normal	Abnormal/Mosaic
LTC-villi	Normal	Abnormal/Mosaic	Abnormal/Mosaic
Fetus or Newborn	Normal	Normal	Normal

Unfortunately, the situation is not always that simple. The distribution of different cell lines among the different tissues is not necessarily equal (Ledbetter et al., 1992). In 1994, Pittalis et al. proposed a more detailed classification of all theoretical types of karyotypic combinations in the trophoblast (STC-viili), mesenchymal core of the villi (LTC-villi), and the fetus proper (Table 3). This classification includes all possibilities of discrepancy among the three compartments STC-villi, LTC-villi and fetus.

Table 3 - Classification of theoretical combinations of mosaicisms according to Kalousek et al. (1992) and Pittalis et al. (1994)

	Category	STC-villi	LTC-villi	Fetus
Homogeneous				
Normal	NHC	-	-	-
Abnormal	AHC	+	+	+
Mosaicism				
Generalized	GMAC	m	m	m
Concordant	GMRC	+	+	m
		+	m	m
		m	m	+
		m	+	+
		+	m	+
		m	+	m
Discordant	GMTD	+[1]	+[2]	+[3]
	GMDD	-	+	+
		-	m	m
		-	+	m
		-	m	+
	GMDC	+	-	+
		m	-	m
		+	-	m
		m	-	+
Confined				
Placenta	CPM I	+	-	-
	CPM I	m	-	-
	CPM II	-	+	-
	CPM II	-	m	-
	CPM III	+	+	-
	CPM III	m	m	-
	CPM III	+	m	-
	CPM III	m	+	-
	CPM III	+[1]	+[2]	-
Fetus	CFM	-	-	+
		-	-	m

STC = short-term culture; LTC = long-term culture; CPM = confined placental mosaicism;
- = normal karyotype; + = abnormal karyotype; m = mosaic karyotype; 1,2,3 = different abnormal karyotypes.

NHC = Normal homogeneous concordance
AHC = Abnormal homogeneous concordance
GMAC = Generalized mosaicism absolute concordance
GMRC = Generalized mosaicism relative concordance
GMTD = Generalized mosaicism total discordance
GMDD = Generalized mosaicism confined direct normality
GMDC = Generalized mosaicism confined culture normality
CFM = Confined fetal mosaicism

Knowledge of the early embryonic development is essential to understand the background of the theoretical consistencies of mosaicism.

A model on the first postzygotic cell divisions has been proposed by Crane and Cheung (1988) and Bianchi (1993). From the 8 cell to the 16 cell stage, the formation of *the morula* starts with the separation of the cells into two compartments: (1) an outer cell layer, the *trophoblast,* which gives rise to the trophoblast part (cytotrophoblast and syncytiotrophoblast) of the placenta and (2) an *inner cell mass* (ICM) which gives rise to the embryo.
Subsequently morphological changes occur in the ICM resulting in two layers: (1) the *epiblast* and (2) the *hypoblast.* This stage of development is known as the *blastocyst stage* (≥64 cells) which contributes to the forming of the embryo and several extra-embryonic structures. The epiblast gives rise to all or nearly all of the cells of the embryo and the hypoblast gives rise to the extra embryonic mesoderm (EEM). The EEM includes the yolk sac, mesoderm of amniotic and chorionic membranes, umbilical cord, and mesodermal core of chorionic villi. The latter component is investigated in the LTC-villi.
Concurrently, the trophoblast cells start to proliferate rapidly and gradually differentiate into two layers: (1) an inner *cytotrophoblast,* which is mitotically active (mitoses can be seen and studied in STC-villi), and (2) an outer *syncytiotrophoblast* (Figure 4 and 5).

1.4.3 *Mosaicism for numerical chromosome aberrations*

Mosaicism occurs through errors in either meiosis or mitosis. In case of a meiotic error in the gamete, this will lead to the formation of a trisomic zygote. During early embryonic development the extra chromosome can be removed; this reduction of trisomy to disomy is known as 'trisomic zygote rescue'. Theoretically, there are three possibilities for this rescue, leading to either a normal morula or to a mosaic morula when it happens in the first postzygotic cell division: 1) chromosome demolition, which involves the destruction and removal of one of the three chromosomes resulting in two disomic daughter cells, 2) non-disjunction, where a failure of the duplication product will produce one disomic and one lethal quadrisomic cell, 3) anaphase lagging, where one duplication product stays behind and gets lost during mitosis, resulting in one disomic and one trisomic daughter cell (Figure 6)(Los et al., 1998a; Van Opstal, 1998).
If the 'trisomic zygote rescue' is delayed to a second or subsequent cell division, the morula will always be mosaic; the later the rescue takes place the more abnormal cells are present in the morula.

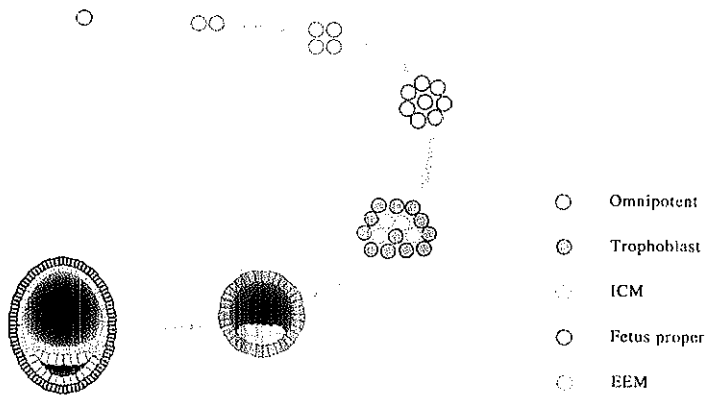

Figure 4 illustrates the normal early embryonic development and shows that the cells obtained by CVS do not belong to the compartment of the fetus proper as do the amniotic fluid cells obtained by amniocentesis

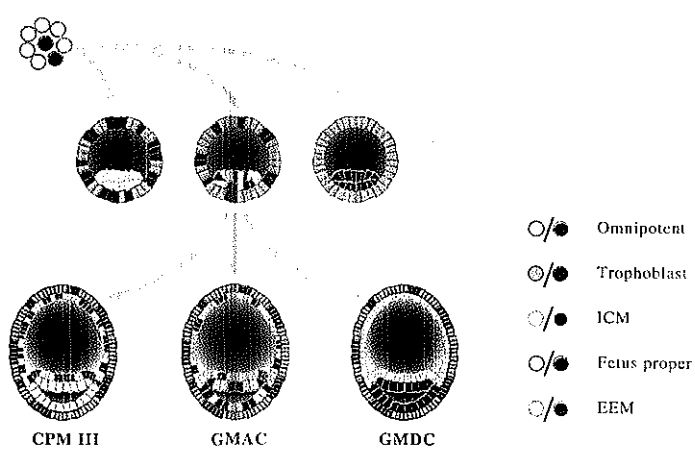

Figure 5 demonstrates an example of possible distribution of normal and abnormal cells in case of mosaicism leading to theoretical combinations according to Pittalis et al. (1994)

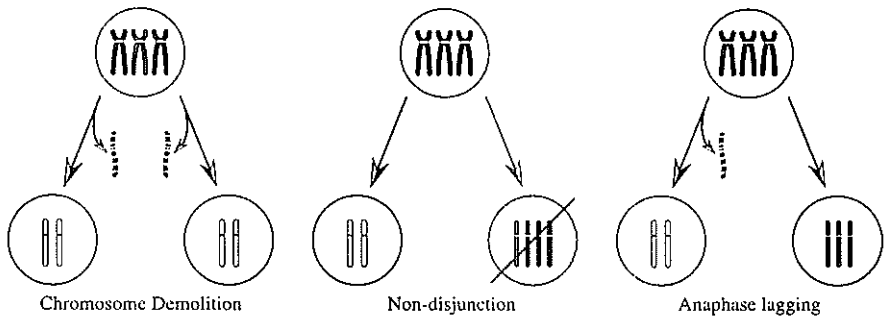

Figure 6 - Theoretical possibilities of trisomic zygote rescue

In case of a mitotic error, this can also lead to a mosaicism. Depending on the timing of mitotic non-disjunction the morula will be completely abnormal or mosaic. An example is given in Figure 6a. Theoretical distributions of normal (disomic) and abnormal (trisomic) cells after mitotic non-disjunction are demonstrated in Figure 6a when this event happens in the first four cell divisions. If the mitotic error occurs in the first cell division this will lead to a homogeneous abnormal morula while there will be a mosaic morula when this event happens during any subsequent cell division.

1.4.4 Mosaicism for structural chromosome aberrations

Not only a numerical mosaicism causes a diagnostic dilemma, but also the finding of a structural mosaicism. The mitotic origin of a structural rearrangement, a deletion, is given in Figure 7, and the theoretical distribution of normal and abnormal cells following a mitotic event in one of the first four cell divisions is given in Figure 7a.

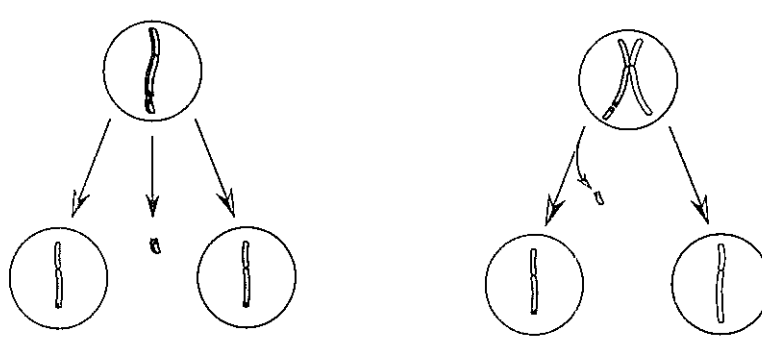

Figure 7 - Mitotic origin of a structural rearrangement; a deletion

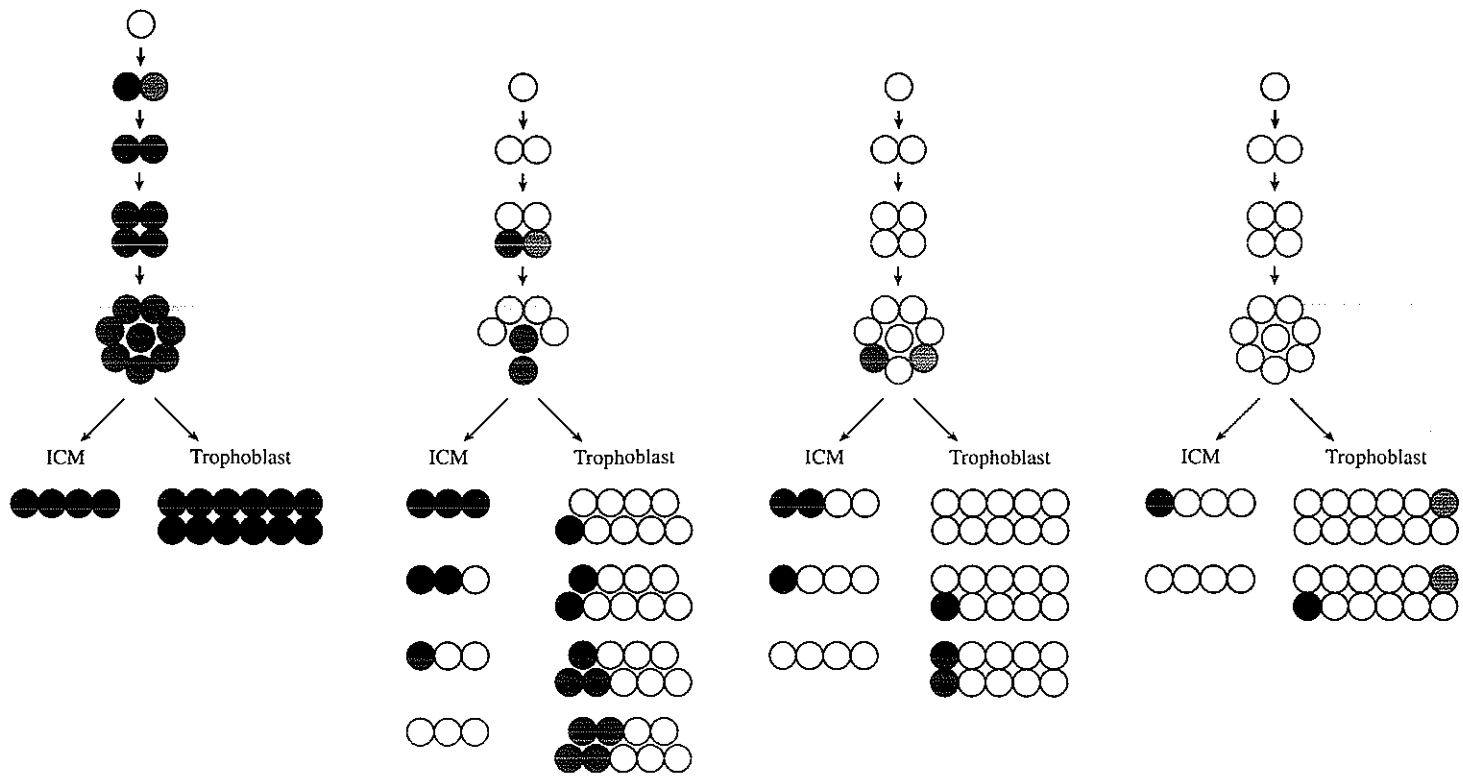

Figure 6a - Distribution of normal and abnormal cells after mitotic non-disjunction in one of the first four cell-divisions; one possibility of compensatory reallocation in the case of cell-loss is shown. ○ = disomic; ● = trisomic; ◍ = monosomic.

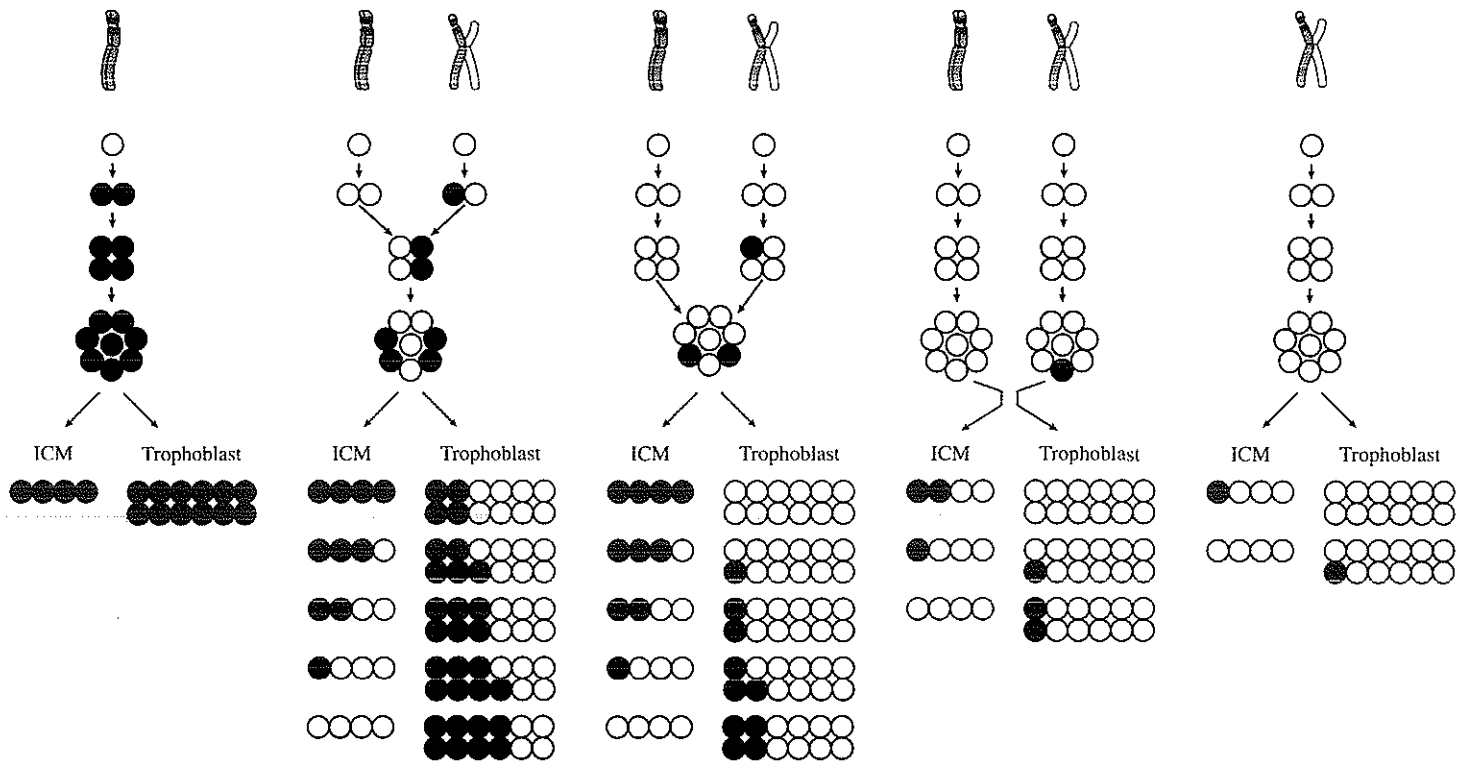

Figure 7a - Distribution of normal and structurally abnormal cells following a mitotic event in one of the first four divisions, affecting both or only one of the daugther cells. ○ = disomic; ● = deletion.

Looking at Figure 6a and 7a it is clear that differentiation between generalized mosaicism and CPM would be desirable. This might be done by a work-up protocol, similar to the work-up protocol used for the differentiation between true mosaicism and pseudomosaicism in amniotic fluid cells.
In 1993, we started with the development of a work-up protocol for the differentiation between generalized mosaicism and CPM in case of STC-villi analysis only (Table 4)(Los et al., 1998b). After the introduction of routine analysis of LTC-villi additional to STC-villi in 1997, we developed a second work-up protocol for the analysis of both STC- and LTC-villi (Table 5)(Van den Berg et al., 2000). Both work-up protocols turned out to be very useful guidelines for the differentiation between generalized mosaicism and CPM.

Table 4 - The number of metaphases to be investigated in STC-villi only (LTC-villi not available)

		STC-villi	Follow-up amniocentesis	
		no. of cells	**FISH**	**Karyotype**
Normal karyotype		16		
Numerical aberration				
+21		16	-	-
Triploidy		12	-	-
47,XXY 47,XXX 47,XYY		16	+	-
+13, +18				
with US abnormality		16	-	-
without US abnormality		16	+	-
45,X				
with US abnormality		16	-	-
without US abnormality		16	+	-
Aneuploidy ≠13, 18, 21, X and Y		16	+	-
Mosaic aneuploidy		20	+	-
Mosaic tetraploidy		30	consideration in each case	
One cell abnormality +7,+8,+9,+11,+13,+14,+15, +18,+21,+mar		30	-	-
Other			consideration in each case	
Structural rearrangement				
Familiar balanced		16	-	-
Familiar unbalanced		16	-	-
De -novo	100%	16	-	+
	mosaic	20	-	+

FISH = FISH on uncultured amniotic fluid-cells

Table 5 - Work-up protocol for the investigation of STC-and LTC -villi, in various circumstances

	STC-villi (n)	LTC-villi (n)	LTC-villi result	Cytogenetic interpretation	AC
Normal karyotype in STC-and LTC-villi	8	8	-	**NHC**	-
Numerical aberration in STC-villi					
Trisomy 21	16	-	-	**AHC**	-
Trisomy 13, 18 with US abnormality	16	-	-	**AHC**	-
without US abnormality	16	8	Abn Mos Norm	AHC, GMRC, CPM III **GMRC**, CPM III GMDC, **CPM I**	- + ?
Triploidy	12	-	-	**AHC**	-
47,XXY, 47,XXX, 47,XYY	16	8	Abn Mos Norm	AHC, GMRC, CPM III **GMRC, CPM III** GMDC, **CPM I**	- + ?
45,X with US abnormality	16	8	Abn Mos Norm	AHC, **GMRC**, CPM III **GMRC**, CPM III GMDC, **CPM I**	- - ?
without US abnormality	16*	8*	Abn Mos Norm	AHC, GMRC, CPM III **GMRC, CPM III** GMDC, **CPM I**	- + -
Aneuploidy ≠13, 18, 21, X and Y	16	8	Abn Mos Norm	AHC, **GMRC, CPM III** GMRC, **CPM III** GMDC, **CPM I**	+ + -
Mosaic aneuploidy	20*	10*	Abn Mos Norm	**GMRC**, CPM III **GMAC, GMRC, CPM III** GMDC, **CPM I**	? + -
One cell abnormality (+7, 8, 9, 11, 13, 14, 15, 18, 21, ESAC)	20	10	-	**NHC**	-
Tetraploidy	special protocol ▲				
Structural rearrangement in STC-villi					
Familial balanced	8	8	-	**AHC**	-
unbalanced	16	-	-	**AHC**	-
De-novo balanced	16	8	Abn Mos Norm	AHC, GMRC, CPM III GMRC, CPM III GMDC, **CPM I**	- - -
unbalanced	16	8	Abn Mos Norm	AHC, GMRC, CPM III GMRC, CPM III GMDC, **CPM I**	- - -
ESAC familial	16	16	Abn Mos Norm	AHC, GMRC, CPM III **GMRC**, CPM III GMDC, CPM I	- - -
De-novo	16	8	Abn Mos Norm	AHC, GMRC, CPM III **GMRC, CPM III** GMDC, **CPM I**	- + -
Mosaic structural rearrangement	20	10	Abn Mos Norm	**GMRC**, CPM III **GMAC, GMRC**, CPM III GMDC, **CPM I**	- - -

Table 5 - continued

	STC-villi (n)	LTC-villi (n)	STC-villi result	Cytogenetic interpretation	AC
Normal karyotype in STC-and LTC-villi	8	8			-
Numerical aberration in LTC-villi with normal STC-villi					
One cell abnormality	8	16		NHC	-
100%	8*	16*	Mos	GMRC, CPM III	-
			Norm	GMDD, CPM II	+
Mosaic	8*	16*	Mos	GMAC, GMRC, CPM III	+
			Norm	GMDD, CPM II	?
Structural rearrangement in LTC-villi with normal STC-villi					
One cell abnormality	8	8		NHC	-
100%	16	16	Mos	GMRC, CPM III	+
			Norm	GMDD, CPM II	+
Mosaic	16	16	Mos	GMAC, GMRC, CPM III	+
			Norm	GMDD, CPM II	-

* = additional FISH on interphase level; ▲ = previously described by Noomen et al. (2000); Van den Berg et al. (2000), Abn = abnormal; AC = amniocentesis; ESAC = Extra Structural Abnormal Chromosome; Mos = mosaic; Norm = normal; for abbreviations see also Table 3.

Cytogeneticists spend a large amount of time distinguishing pseudomosaicism from true mosaicism and confined placental mosaicism from generalized mosaicism, and often feel uncomfortable interpreting the findings.
The guidelines introduced by Hsu et al. in 1992 for diagnosing pseudomosaicism and true mosaicism in amniotic fluid cell cultures and our own guidelines for the distinguishing of confined placental mosaicism and generalized mosaicism in chorionic villi has been considered very efficient and effective. Both guidelines will not only give more insight into the frequency of mosaicisms but will also lead to a better interpretation of the cytogenetic results. This will lead to a higher predictive value of cytogenetic diagnosis after amniocentesis and chorionic villus sampling which also means a decrease in the number of follow-up investigations.

1.5 Aims of this thesis

The general principles of our prenatal investigations are:

- To establish a reliable diagnosis which means a sensitivity and specificity at or as close as possible to 100%.
- To establish the diagnosis as early as possible with the safest technique available and with the maximum yield of relevant information.

In our laboratory, we have regularly been confronted with diagnostic problems after CVS and to a lesser extent after amniocentesis.
We decided to evaluate our CVS and amniocentesis analyses for reliability, accuracy and efficiency in order to arrive at an optimal quality of prenatal cytogenetic service.

- Since the introduction of CVS we have occasionally (1-2%) been confronted with diagnostic problems after the analysis of STC-villi alone. This, together with reported collaborative studies has raised some concerns about the accuracy and reliability of CVS after the analysis of STC-villi alone. The evaluation of 3500 CVS cases is addressed in *Chapter* 2.
- Recommendations about the combined use of both STC- and LTC-villi were made to provide a more accurate diagnosis in chorionic villi. We studied the reliability of cytogenetic results in both villi compartments and compared the number of follow-up amniocenteses with the figure from the study of STC-villi alone. Results of this study are presented in *Chapter* 3.
- Multiple gestations present a particular problem in prenatal diagnosis. Most reports concern the increased risk of fetal loss for women undergoing amniocentesis. However, the genetic and/or laboratory aspects of amniocentesis and CVS were not explored in depth of any large series. We investigated the accuracy and reliability of both procedures to arrive at better recommendations for this group of patients. Our experience with 500 multiple pregnancies is presented in *Chapter 4*.
- Since first-trimester CVS has not reached the popularity of second-trimester amniocentesis despite its clinical and psychological advantages questions have been raised as to why this is the case. Differences in sampling related risks, or in the reliability and accuracy of laboratory results in favour of amniocentesis are possible causes. We have evaluated the diagnostic performance of cytogenetic investigations after amniocentesis and CVS as a potential cause for this remarkable observation. This study is described in *Chapter 5*.
- Attention was also given to (recurrent) specific diagnostic cytogenetic problems. An evaluation of some special cases are presented in *Chapter 6*, such as the finding of trisomy 9 which poses a serious problem in prenatal diagnosis (6.1); the meaning and interpretation of mosaic tetraploidy in CVS (6.2); a presumed case of GMDC which turned out to be a general mosaicism after extensive investigations (6.3); the finding of the simultaneous presence of isochromosomes 18p and 18q, and the determination of the mechanism of formation is presented in 6.4.

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Chapter 2

Cytogenetic investigation

in STC-villi

ABNORMAL KARYOTYPES IN SEMI-DIRECT CHORIONIC VILLUS PREPARATIONS OF WOMEN WITH DIFFERENT CYTOGENETIC RISKS

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Summary

Among 3499 cytogenetically investigated semi-direct chorionic villus samples, 219 (6.3%) abnormal karyotypes were encountered. The karyotypes were considered certainly abnormal (generalized abnormal with high probability) in 109 cases (3.1%), and in 110 cases (3.1%) uncertainly abnormal (potentially confined to the placenta), requiring further investigation. Of these 110 uncertain abnormalities, the cytogenetic result turned out to be finally abnormal representing generalized abnormality in 36 cases (32.7%), finally normal representing confined placental mosaicism (CPM) in 69 cases (62.7%), and remained undetermined in 5 instances (4.5%). The rate of the numbers of certainly abnormal and all (certainly + uncertainly) abnormal results, the certainty rate, and that of generalized abnormalities and all abnormalities (generalized abnormalities + CPM cases), the predictive value, are strongly correlated with the cytogenetic risk. Therefore, we advise chorionic villus sampling for cytogenetic investigation only in women with a cytogenetic risk equal to or exceeding that of a 40-year-old pregnant women. Because of the high rate of prenatal follow-up investigations after the finding of uncertain results in semi-direct villi, semi-direct and cultured villi should be karyotyped simultaneously .

Introduction

First-trimester prenatal diagnosis in chorionic villi was successfully introduced in our department in 1983 (Sachs et al., 1985; 1988). However, since 1992, the number of chorionic villi displays a decrease with a concomitant increase in the number of amniotic fluid samples (Fig. 1). There are two reasons for this decrease. The first concerns the yet unsolved problem of inducing fetal vascular disruptive syndromes by the sampling procedure (Firth et al., 1994; 1996; NICHD, 1993; Kuliev et al., 1996; Olney et al., 1995; Froster & Jackson, 1996, Los et al., 1996). The second reason is the limited representativity of an abnormal karyotype in chorionic villi for the actual fetal karyotype due to confined placental mosaicism (CPM) (Kalousek & Dill, 1983; Kalousek & Vekemans 1996; Wolstenholme, 1996). Various studies focused on the combined cytogenetic results in cytotrophoblast cells, direct or semi-direct (short term culture; STC) villi, and cultured cells of the mesenchymal core, cultured (long term culture; LTC) villi (Vejerslev & Mikkelsen, 1989; Teshima et al., 1992; Ledbetter et al., 1992; Smidt-Jensen et al., 1993; Wang et al., 1993; Association of Clinical Cytogeneticists Working Party on Chorionic Villi in Prenatal Diagnosis, 1994; Wolstenholme et al., 1994; Pittalis et al., 1994). Some studies comprised cytogenetics in STC-villi only (Caspari et al., 1994; Leschot et al., 1996), others in LTC-villi only (Hogge et al., 1986; Fryburg et al., 1993).

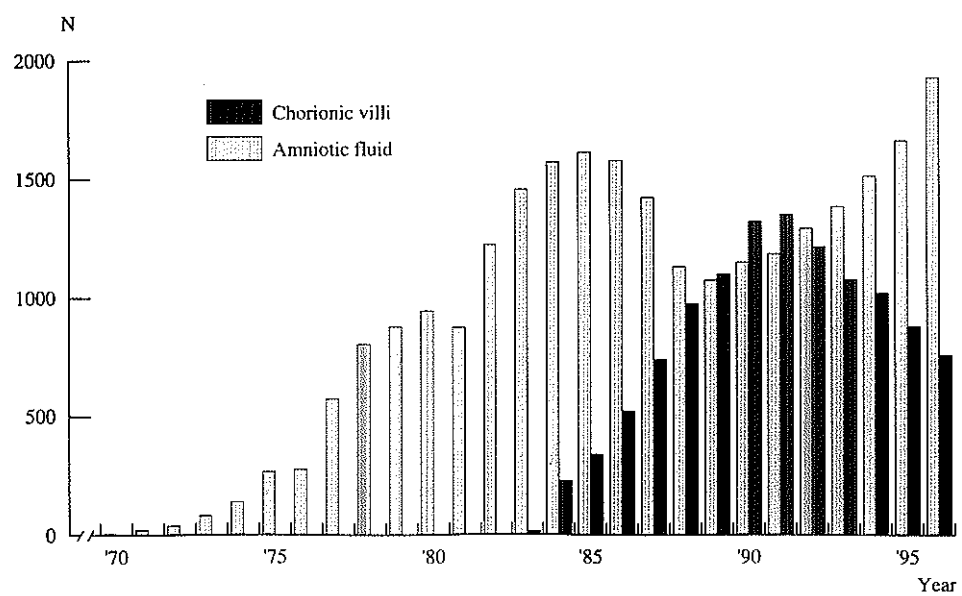

Figure 1 - The numbers of amniotic fluid and chorionic villus samples received from 1970 until 1996

The best results of karyotyping in chorionic villi are realized when STC- and LTC-villi are investigated simultaneously (Ledbetter et al., 1992; Association of Clinical Cytogeneticists Working Party on Chorionic Villi in Prenatal Diagnosis, 1994; Pittalis et al., 1994). However, for technical or economic reasons various restrictive policies concerning the use of either STC- or LTC-villi alone have been implemented. Such a restrictive policy of discarding STC-villi and using only LTC-villi has been reported by Smidt-Jensen et al. (1993). Another restrictive policy is the routine cytogenetic investigation in STC-villi, and in case of an abnormality the additional investigation of LTC-villi, which has also been implemented in our laboratory for some years (Breed et al., 1990; Sachs et al., 1990; Leschot et al., 1996).
We stopped the culturing and potential investigation of LTC-villi in 1993 since less than 1% of the initiated cultures were actually investigated and in the majority of cases, LTC-villi turned out to be non-available when needed. We present our experiences with prenatal cytogenetic diagnosis in STC-villi during a four year period.

Material and Methods

During the years 1993-1996, 3726 chorionic villus samples were received; 3027 samples were from the Dijkzigt Hospital, Rotterdam, The Netherlands, and 450 from the Merwede Hospital, Dordrecht, The Netherlands. The remaining samples came from other centres in The Netherlands or from other countries. Chorionic villus sampling in the Dijkzigt and Merwede Hospitals was performed transabdominally in all cases as decribed earlier (Jahoda et al., 1990). For the samples coming from other centres, information on the method of sampling was not provided in most cases. The indication for prenatal diagnosis was cytogenetic in 3303 of the 3726 cases [advanced maternal age ($\geq$ 36 years), parental carriership for structural rearrangements or marker chromosomes, fetal abnormalities on ultrasound, recurrence risk of chromosomal abnormality, previous child with multiple congenital malformations, family history of chromosomal abnormality, risk for X-linked diseases]. In the other 423 cases, the reason for prenatal diagnosis was biochemical (recurrence risk for various metabolic diseases, risk for X-linked metabolic disease in male fetuses) or DNA-investigation (recurrence risk for various autosomal recessive, autosomal dominant or X-linked inherited diseases, previous child with microdeletion or uniparental disomy syndrome). In 206 of these 423 cases, chromosomal analysis was additionally performed.

STC-villi slides were prepared using fluorodeoxyuridine (FdU) synchronization (Gibas et al., 1987). Karyotyping was routinely performed with Trypsin-Giemsa staining. Sometimes other staining techniques, such as DA-DAPI-staining, Ag-NOR-staining, C-banding or endonuclease-banding were used. Normally 16 cells were analysed, in the case of a single cell with trisomy 8, 9, 13, 18 or 21, and in the case of a supernumerary marker chromosome this number was extended to 30 cells. When two or more cells among the 16 analysed cells showed the same abnormality, at least 30 cells were investigated whenever possible. A karyotype in STC-villi was

considered normal in the case of 46,XX or 46,XY, ± inversion (9)(p12q13), and/or ± one cell exhibiting any abnormality. All other results were considered abnormal. Abnormal karyotypes were considered certainly abnormal when they were assumed to represent generalized abnormality with a high probability and allowed for clinical decisions without further prenatal investigations. A cytogenetic result was considered uncertainly abnormal when it could potentially be confined to the placenta, and required further investigation (Ledbetter et al., 1992; Association of Clinical Cytogeneticists Working Party on Chorionic Villi in Prenatal Diagnosis, 1994; Leschot et al., 1996). A cytogenetic result was defined as a mosaicism when two or more cells showed a karyotype different from the karyotype(s) in the other cells (cell lines). Mosaicism was divided into three levels: low level (≤ 10% abnormal cells), medium level (11% - 33.3% abn cells) and high level (≥ 33.3% abn cells).

Follow-up studies involved dependend on the encountered cytogenetic problem fluorescence *in situ* hybridization (FISH) on STC-villi slides and sometimes subsequently on (uncultured) amniotic fluid cells, parental karyotyping, amniocentesis and/or ultrasound investigation. FISH on interphase cells of STC-villi preparations displaying aneuploidy or polyploidy mosaicism was used for differentiation of coincidental local mitotic division errors from mosaicism and was performed as decribed before (Van Opstal et al., 1995; 1998).
In the case of confirmation of the mosaicism in STC-villi, FISH was performed with the same probe(s) on uncultured amniotic fluid cells for the differentiation between CPM and generalized mosaicism (Van den Berg et al., 1997; Van Opstal et al., 1998). Interpretation of signal distributions has been described previously (Van Opstal et al., 1998). FISH on metaphases of STC-villi and cultured amniotic fluid cells was carried out for potential identification of marker chromosomes and derivatives (In 't Veld et al., 1995a; Joosten et al., 1997). Parental karyotypes were prepared from peripheral blood lymphocytes according to standard techniques. Amniotic fluid cells were cultured with the *in situ* method on glass coverslips also according to standard techniques. First- and second-trimester ultrasound investigations were performed in our department as described previously (Wladimiroff et al., 1995; Cha'ban et al., 1996). In some cases with (mosaic) unusual trisomy, DNA studies were performed for the investigation of the parental origin of the supernumerary chromosome and/or on uniparental disomy (UPD) (Van den Berg et al., 1997; Van Opstal et al., 1998).

In the case of termination of pregnancy (TOP), we always tried to confirm the cytogenetic abnormality in fetal fibroblasts. Information on the course and outcome of pregnancy was received from the women and/or the referring midwife or physician. Birthweights of babies were appreciated according to the tables of Kloosterman (1970). Statistical analysis comprised χ^2 tests and binomial statistics.

Results

STC-villi preparations were made of 3509 chorionic villus samples. In 10 cases (0.3 %), no cytogenetic diagnosis could be made due to laboratory failure. In 3499 cases

(99.7%), a cytogenetic result was achieved. Normal karyotypes were found in 3280 samples (93.7%) and abnormal results in 219 cases (6.3%). Single-cell abnormalities were encountered in 566 samples (16.2%). Half of the abnormal karyotypes were certainly abnormal (N=109), and the other half uncertainly abnormal (N=110); the exact nature and numbers of the certainly and uncertainly abnormal cytogenetic results in relation to the indication for prenatal diagnosis are shown in Fig. 2. In one case, an unbalanced rearrangement was considered certain on the indication ultrasound abnormalities, because it became known after sampling that there was a familial rearrangement in the woman's family of which the encountered rearrangement was one of the possible unbalanced forms. The distribution of certainly and uncertainly abnormal cytogenetic results in STC-villi among the various indications is presented in Table 1. The frequencies of certainly abnormal cytogenetic results in the various indications showed a statistically significant difference, in contrast to those of uncertainly abnormal results. The certainty rates (certainly abnormal:all abnormal) differed statistically significantly between the various indications.

Table 1 - Certainty rates of abnormal cytogenetic results (certainly abnormal: all abnormal) in STC-villi among the various indications

Indication	N	Normal	Abnormal			Certainty rate[3] (95% CI)
			N	Certainly[1]	Uncertainly[2]	
MA 36-39	2282	2191	91 (4.0%)	13 (0.6%)	78 (3.4%)	0.143 (0.078 - 0.232)
MA ≥ 40	447	424	23 (5.1%)	13 (2.9%)	10 (2.2%)	0.565 (0.345 - 0.768)
US ABN	165	116	49 (29.7%)	40 (24.2%)	9 (5.5%)	0.816 (0.680 - 0.912)
CARRIER	80	41	39 (48.8%)	39 (48.8%)	0 (0%)	1.000 (0.910 - 1.000)
DNA/BIO	206	201	5 (2.4%)	2 (1.0%)	3 (1.5%)	0.235 (0.068 - 0.499)
OTHER	319	307	12 (3.8%)	2 (0.6%)	10 (3.1%)	
TOTAL	3499	3280	219 (6.3%)	109 (3.1%)	110 (3.1%)	0.498 (0.432 - 0.564)

95% CI = 95% confidence interval.[1] Frequencies of certainly abnormal results (US ABN and CARRIER combined) differ significantly ($\chi^2 = 727.65$, df = 4, $p < 0.001$). [2] Frequencies of uncertainly abnormal results (US ABN and CARRIER combined) do not differ significantly ($\chi^2 = 3.85$, df = 4, $p > 0.05$). [3] Certainty rates differ significantly ($\chi^2 = 110.12$, df = 4, $p < 0.001$).

Follow-up investigations and pregnancy outcomes in women with uncertainly abnormal karyotypes in STC-villi and a final abnormal prenatal cytogenetic diagnosis representing generalized abnormality (N=36) are presented in Table 2. TOP was requested and carried out in 23 cases; in 12 cases, the pregnancies were continued because none or only minor phenotypic consequences were expected from the abnormal karyotypes.

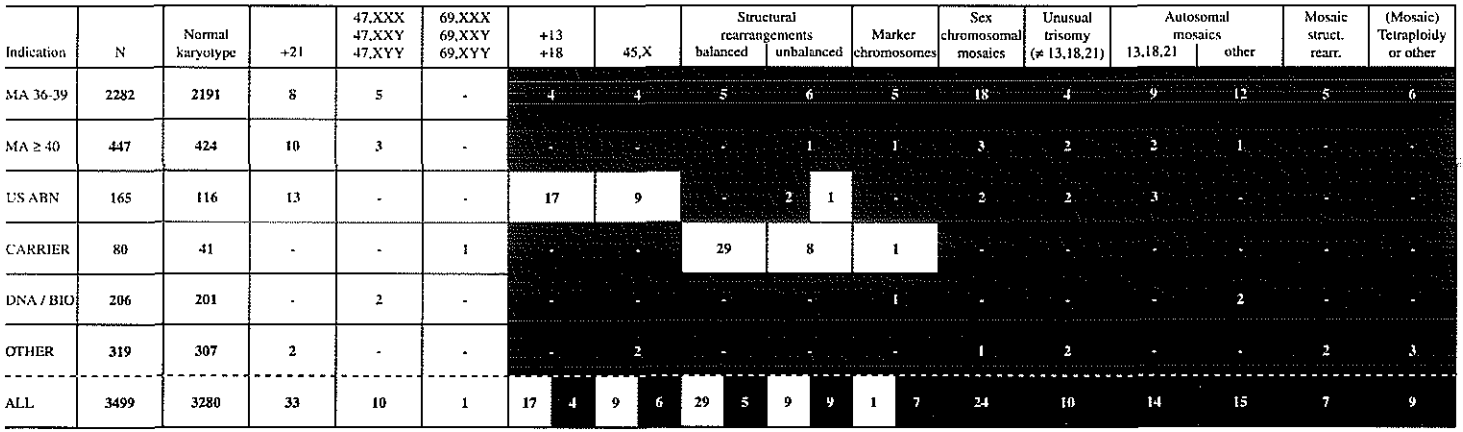

Figure 2 - Certainly (white squares) and uncertainly (black squares) abnormal karyotypes in STC-villi of women with various indications for prenatal cytogenetic diagnosis during the study period 1993-1996. MA = maternal age; US ABN = ultrasound abnormalities; CARRIER = parental carriership for structural rearrangement or marker chromosome; DNA/BIO = cytogenetic investigation secondary to DNA or biochemical investigation; OTHER = previous child with chromosome abnormality or congenital malformations, family history of chromosome abnormality, other reasons for prenatal cytogenetic diagnosis; struct. = structural; rearr. = rearrangement

Table 2 - Further investigations in cases of uncertainly abnormal results in STC-villi with finally abnormal cytogenetic results (generalized abnormalities). Abnormal or N at FISH means an abnormal or normal signal distribution. No comment at pregnancy outcome means a normal baby with a birthweight between *p5* and *p95*

Case No.	PD indication	Karyotype in STC-villi	FISH on STC-villi	Parental karyotype	Ultrasound investigation	Amniocentesis		Pregnancy outcome	
						FISH	Karyotype		
1	MA 36-39	47,XX,+18[18]	Abnormal	-	N	Abnormal	47,XX,+18	TOP	47,XX,+18
2	MA 36-39	47,XY,+18[30]	Abnormal	-	N	Abnormal	47,XY,+18	TOP	-
3	MA 36-39	47,XY,+18[30]	-	-	-	-	-	TOP	47,XY,+18
4	MA 36-39	47,XX,+21[28]/46,XX[2]	Abnormal	-	Abnormal	-	-	TOP	47,XX,+21
5	MA 36-39	47,XX,+21[24]/46,XX[6]	Abnormal	-	N	Abnormal	47,XX,+21	TOP	47,XX,+21
6	MA 36-39	47,XX,+21[28]/46,XX[2]	-	-	-	-	-	TOP	47,XX,+21
7	MA ≥ 40	47,XY,+18[27]/46,XY[3]	Abnormal	-	N	Abnormal	-	TOP	47,XY,+18
8	US ABN	47,XX,+21[27]/46,XX[3]	-	-	-	-	-	TOP	47,XX,+21
9	US ABN	47,XY,+18[18]/ 48,XY,+18,+20[13]/ 46,XY[3]	Abnormal	-	-	Abnormal	47,XY,+18	TOP	47,XY,+18
10	US ABN	47,XX,+21[27]/46,XX[3]	Abnormal	-	-	-	-	TOP	47,XX,+21
11	MA 36-39	45,X[10]	-	-	N	-	45,X[1]/46,XX[54]	Cont.	♀, < p2.3
12	MA 36-39	45,X[22]	-	-	Abnormal	-	-	TOP	45,X
13	MA 36-39	45,X[30]	Abnormal	-	Abnormal	Abnormal	45,X	TOP	45,X
14	OTHER	45,X[16]	Abnormal	-	N	N	45,X[5]/46,XX[34]	Cont.	♀
15	OTHER	45,X[16]	Abnormal	N,N	Abnormal	-	-	TOP	45,X
16	MA 36-39	45,X[31]/46,XY[24]	Abnormal	-	-	Abnormal	45,X[3]/46,XY[14]	TOP	45,X[5]/46,XY[45]
17	MA 36-39	46,Xdel(Y)[16]/ 46,Xidic[Y][14]/45,X[4]	Identification	N,N	N	Identification	46,XidicY[17]/ (46,XidicY/delY)[2]/ 45,X[1]	TOP	46,Xidic(Y)(q11)[15]/ 45,X[13]/ 46,Xdel(Y)(q11)[2][1]
18	MA 36-39	47,XXY[6]/46,XY[2]	Abnormal	-	-	Abnormal	47,XXY[3]/46,XY[6]	Cont.	♂
19	OTHER	45,X[6]/46,XX[24]	Abnormal	-	N	Abnormal	45,X[1]/46,XX[15]	Cont.	♀
20	MA 36-39	47,XX,+9[30]	Abnormal	-	Abnormal	Abnormal	47,XX,+9[7]/46,XX[30]	TOP	FISH;47,XX,+9[30%]/ 46,XX [70%][2]
21	US ABN	47,XY,+22[16]	-	-	-	Abnormal	47,XY,+22	TOP	47,XY,+22
22	US ABN	47,XY,+22[16]	-	-	Second Abnormal	-	-	TOP	47,XY,+22
23	OTHER	47,XY,+9[16]	Abnormal	-	Abnormal	Abnormal	47,XY,+9[4]/46,XY[26]	TOP	FISH;47,XY,+9[87%]/ 46,XY[13%][2]

Table 2 - Continued

Case No.	PD indication	Karyotype in STC-villi	FISH on STC-villi	Parental karyotype	Ultrasound investigation	Amniocentesis		Pregnancy outcome	
						FISH	Karyotype		
24	MA 36-39	46,XX,add(1q)[16]	Identification	N,N	-	-	-	TOP	46,XX,dup(1)(q2?3q3?2)
25	MA 36-39	46,XX,der(21;21) (q10;q10),+21[16]	Abnormal	-	N	Abnormal	46,XX,der(21;21) (q10;q10),+21	TOP	46,XX,der(21;21) (q10;q10),+21
26	MA 36-39	46,X,der(Xp)[16]	Identification	N,N	N	Identification	46,X,inv(X)(p22.3q26)	Cont.	♀
27	MA 36-39	46,XX,inv(3) (p21q26.2)[16]	-	46,XY,inv(3) 46,XX	-	-	-	Cont.	♀
28	MA 36-39	46,XX,t(4;13) (q34;q21.3)[16]	-	46,XY,t(4;13) 46,XX	-	-	-	Cont.	♀
29	MA 36-39	46,XY,inv(8) (p21.3p23.3)[16]	-	46,XY,inv(8) 46,XX	-	-	-	Cont.	♂
30	MA 36-39	45,XY,der(13;14) (q10;q10)[16]	-	46,XY 45,XX,der(13;14)	-	-	-	Cont.	♂
31	US ABN	46,XX,der(21;21) (q10;q10),+21[16]	-	-	-	-	-	TOP	46,XX,der(21;21) (q10;q10),+21
32	MA 36-39	47,XX,+mar[28]/ 46,XX[2]	Identification (?)	N/N	-	-	-	TOP	47,XX,+mar[13]/ 46,XX[21]
33	MA 36-39	47,XY,+mar[24]/ 46,XY[14]	Identification (?)	46,XY 47,XX,+mar[21]/ 46,XX[28]	-	-	-	Cont.	♂
34	MA ≥ 40	47,XY,+mar[30]	Identification (satellites)	N,N	N	-	47,XX,+mar	Cont.	♀
35	MA 36-39	92,XXYY[5]/46,XY[45]	-	-	N	-	92,XXYY[2]/ (92,XXYY/46,XY)[2]/ 46,XY[29]	Cont.	♂
36	MA 36-39	45,XX,-21[21]	Identification	46,XY[68]/ 47,XY,+mar[2]/ 46,XX	Abnormal	-	-	IUD	45,XX,-21[3]

Abbreviations(see also legend of Fig. 2): add = additional (chromosomal material); cont. = continuation of pregnancy; del = deletion; der = derivate; idic = isodicentric; inv = inversion; IUD = intra-uterine death; mar = marker chromosome; MCA = multiple congenital malformations; N = normal; NI = non-informative; PD = prenatal diagnosis; RhC = Rhesus factor C; TOP = termination of pregnancy; sel. TOP = selective TOP.[1] Previously described (in 't Veld et al., 1995a); [2] previously described (Van den Berg et al., 1997); [3] previously described (Joosten et al., 1997).

Table 3 - Further investigations in cases of uncertainly abnormal results in STC-villi with finally normal cytogenetic results (CPMs). Abnormal or N at FISH means an abnormal or normal signal distribution. No comment at pregnancy outcome means a normal baby with a birthweight between *p*5 and *p*95

Case No.	PD indication	Karyotype in STC-villi	FISH on STC-villi	Parental karyotype	Ultrasound investigation	Amniocentesis			Pregnancy outcome
						FISH	Karyotype		
37	MA 36-39	48,XY,+13,+20[3]/46,XY[27]	NI	-	N	N	46,XY	Cont.	♂, 34 weeks solutio placentae[1]
38	MA 36-39	47,XY,+13[3]/46,XY[27]	NI	-	N	NI	46,XY	Cont.	♂, < *p*5
39	MA 36-39	47,XX,+18[2]/46,XX[28]	N	-	N	N	46,XX	Cont.	♀, < *p*5[1]
40	MA 36-39	47,XX,+13[2]/46,XX[28]	-	-	N	-	46,XX	Cont.	♀[1]
41	MA 36-39	47,XY,+13[2]/46,XY[28]	-	-	N	-	46,XY	Cont.	♂[1]
42	MA 36-39	47,XX,+18[2]/46,XX[28]	N	-	-	N	46,XX	Cont.	♀[1]
43	MA ≥ 40	50,XX,+7,+13,+20,+21[3]/46,XX[32]	N	-	N	N	46,XX	Cont.	♀
44	MA 36-39	45,X[15]/46,XX[7]	Abnormal	-	-	N	46,XX	Cont.	♀
45	MA 36-39	45,X[3]/46,XX[29]	-	-	-	N	46,XX	Cont.	♀
46	MA 36-39	45,X[2]/46,XX[28]	N	-	-	-	-	Cont.	♀
47	MA 36-39	45,X[8]/46,XX[27]	Abnormal	-	-	N	46,XX	Cont.	♀
48	MA 36-39	45,X[2]/46,XY[28]	N	-	-	-	-	Cont.	♂
49	MA 36-39	47,XXX[8]/46,XX[22]	Abnormal	-	-	N	46,XX	Cont.	♀[1]
50	MA 36-39	45,X[2]/46,XX[27]	Abnormal	-	-	N	46,XX	Cont.	♀
51	MA 36-39	45,X[3]/46,XX[28]	N	-	-	N	46,XX	Cont.	♀
52	MA 36-39	45,X[2]/46,XX[28]	Abnormal	-	-	N	46,XX	Cont.	♀
53	MA 36-39	45,X[2]/46,XY[33]	N	-	-	-	-	Cont.	♂
54	MA 36-39	45,X[2]/46,XX[28]	N	-	-	-	-	Cont.	♀
55	MA 36-39	45,X[2]/46,XX[28]	N	-	-	-	-	Cont.	♀
56	MA 36-39	45,X[5]/46,XX[25]	Abnormal	-	-	N	46,XX	Cont.	♀
57	MA 36-39	45,X[2]/46,XY[23]	Abnormal	-	-	N	46,XY	Cont.	♂
58	MA 36-39	45,X[2]/46,XY[28]	N	-	-	-	-	Cont.	♂
59	MA ≥ 40	45,X[2]/46,XY[28]	N	-	-	-	-	Cont.	♂
60	MA ≥ 40	45,X[2]/46,XY[28]	N	-	-	-	-	Cont.	♂
61	MA ≥ 40	45,X[2]/46,XX[28]	N	-	-	-	-	Cont.	♂

Table 3 - Continued

Case No.	PD indication	Karyotype in STC-villi	FISH on STC-villi	Parental karyotype	Ultrasound investigation	Amniocentesis			Pregnancy outcome
						FISH	Karyotype		
62	US ABN	45,X[2]/46,XX[18]	-	-	Abnormal	-	46,XX	IUD	♀, 27 weeks,omfalocèle, IUGR
63	MA 36-39	47,XX,+7[30]	Abnormal	-	N	N	46,XX	Cont.	♀, *p5*
64	MA 36-39	47,XY,+16[32]	Abnormal	-	N	N	46,XY	Cont.	♂
65	MA 36-39	47,XY,+3[31]	Abnormal	-	N	N	46,XY	Cont.	♂
66	MA ≥ 40	47,XX,+16[30]	Abnormal	-	N	N	46,XX	Cont.	♀, normal, UPD 16[1]
67	MA ≥ 40	47,XX,+22[50]	Abnormal	-	Abnormal	-	-	IUD	15 weeks, 46,XX, MCA[1]
68	OTHER	47,XX,+16[30]	Abnormal	-	Abnormal	Abnormal	46,XX	IUD	♀, IUGR, 33 weeks, MCA, < *p2.3*[1]
69	MA 36-39	47,XX,+7[8]/46XX[26]	Abnormal	-	N	N	46,XX	Cont.	♀[1]
70	MA 36-39	47,XX,+16[2]/46,XX[28]	Abnormal	-	N	N	46,XX	Cont.	♀[1]
71	MA 36-39	47,XY,+7[21]/46,XY[11]	Abnormal	-	N	N	46,XY	Cont.	♂
72	MA 36-39	47,XX,+7[3]/46,XX[27]	Abnormal	-	N	N	46,XX	Cont.	♀[1]
73	MA 36-39	47,XY,+7[12]/46,XY[18]	Abnormal	-	N	N	46,XY	Cont.	♂[1]
74	MA 36-39	47,XX,+22[2]/46,XX[28]	N	-	N	N	46,XX	Cont.	♀
75	MA 36-39	47,XY,+7[6]/46,XY[24]	Abnormal	-	N	N	46,XY	Cont.	♂[1]
76	MA 36-39	47,XX,+2[4]/46,XX[15]	-	-	-	-	46,XX	Cont	♀[1]
77	MA 36-39	47,XY,+3[7]/46,XY[22]	Abnormal	-	N	N	46,XY	Cont.	♂[1]
78	MA 36-39	47,XY,+8[3]/46,XY[15]	-	-	-	-	-	TOP	♂, 46,XY, MCA
79	MA 36-39	47,XX,+11[5]/46,XX[25]	Abnormal	-	N	N	46,XX	Cont.	♀
80	MA ≥ 40	47,XY,+7[4]/46,XY[10]	Abnormal	-	N	N	46,XY	Cont.	♂
81	DNA/BIO	47,XY,+3[6]/46,XY[24]	Abnormal	-	N	N	46,XY	Cont.	♂[1]
82	DNA/BIO	47,XX,+7[3]/46,XX[30]	Abnormal	-	N	N	46,XX	Cont.	♀, 36 weeks[1]
83	MA 39-39	46,XY,der(7p)[13]	-	N,N	-	-	46,XY	Cont.	♂
84	MA 36-39	46,XY,11q-[17]	-	N,N	-	-	46,XY	Cont.	♂, M. Hirschsprung
85	MA 36-39	46,XY,der(10)[16]	-	N,N	-	-	-	TOP	46,XY
86	MA 36-39	46,XX,der(16q)[22]	-	N,N	-	-	46,XX	Cont.	♀, 32 weeks

Table 3 - Continued

Case No.	PD indication	Karyotype in STC-villi	FISH on STC-villi	Parental karyotype	Ultrasound investigation	Amniocentesis			Pregnancy outcome
						FISH	Karyotype		
87	MA ≥ 40	45,X,der(9p)[2]/46,XX,der(9p)[28]	-	N,N	N	-	46,XX	Cont.	♀, 34 weeks, hydrops fetalis (RhC)
88	US ABN	46,XY,add(16q)[20]	Identification (t(11 or 12);16)	N,N	Abnormal	-	-	TOP	46,XY
89	MA 36-39	47,XY,+mar[3]/46,XY[30]	-	N,N	N	-	46,XY	Cont.	♂, < *p5*
90	MA 36-39	47,XX,+mar[17]/46,XX[3]	Identification (?)	N,N	Abnormal	-	-	TOP	46,XX
91	DNA/BIO	47,XX,+mar[3]/46,XX[27]	-	N,N	-	-	46,XX	Cont.	♀
92	MA 36-39	46,XY,8p-[16]/46,XY[2]	-	N,N	-	-	46,XY	Cont.	♂
93	MA 36-39	46,XX,der(5p)[6]/46,XX[13]	-	N,N	-	-	46,XX	Cont.	♀
94	MA 36-39	46,XY,der(7)[22]/46,XY[14]	Identification (?)	N,N	-	-	46,XY	Cont.	♂
95	MA 36-39	46,XX,der(6)[25]/46,XX[6]	Identification (?)	-	-	-	46,XX	Cont.	♀
96	MA 36-39	46,XX,der(13;13)(q10;q10)[12]/ 46,XX[18]	Abnormal	-	Slightly abnormal	N	46,XX	Cont.	♀
97	OTHER (1 of twin)	46,XX,t(6;13)[6]/46,XX[34]	-	-	-	-	46,XX	Cont.	♀, 34 weeks, *p97.7*, bilateral cleft. (other normal ♀)
98	OTHER	46,XX,+5,der(5;13)(q10;q10)[24]/ 46,XX,+13,der(13;13)[1]/46,XX[5]	-	N,N	N	-	46,XX	Cont.	♀
99	MA 36-39	92,XXYY[16]	Abnormal	-	N	N	46,XY	Cont.	♂
100	MA 36-39	92,XXYY[2]/46,XY[11]	Abnormal	-	N	N	46,XY	Cont.	♂, hypospadia, > *p97.7*
101	MA 36-39	92,XXYY[43]	Abnormal	-	Transient abnormal	N	46,XY	Cont.	♂, > *p95*
102	MA 36-39	92,XXXX[22]/46,XX[14]	Abnormal	-	N	N	46,XX	Cont.	♀
103	OTHER	92,XXYY[20]/46,XY[2]	-	-	N	N	46,XY	Cont.	♂
104	OTHER	92,XXYY[34]/46,XY[53]	Abnormal	-	N	N	46,XY	Cont.	♂
105	OTHER	92,XXYY[5]/46,XY[25]	N	-	Transient abnormal	-	-	Cont.	♂, > *p97.7*

Abbreviations: see Fig. 2 and Table 2.
[1] Previously described (Van Opstal et al., 1998).

Table 4 - Further investigations in cases of uncertainly abnormal results in STC-villi with finally undetermined cytogenetic results. Abnormal or N at FISH means an abnormal or normal signal distribution. No comment at pregnancy outcome means a normal baby with a birth weight between *p*5 and *p*95

Case No.	PD indication	Karyotype in STC-villi	FISH on STC-villi	Parental karyotype	Ultrasound investigation	Amniocentesis		Pregnancy outcome
						FISH	Karyotype	
106	MA 36-39	47,XY,+13[4]	-	-	Abnormal	-	-	IUD, growth failure fetal tissue
107	MA 36-39 (1 of twin)	45,X [30]	Abnormal	N,N	N	Abnormal	Growth failure (12 weeks amniotic fluid)	Sel. TOP of affected twin (other normal ♂)[1]
108	US ABN	45,X[2]/46,XY[28]	Abnormal	-	-	-	-	TOP, no tissue for confirmation
109	MA 36-39	47,XX,+3[13]/46,XX[19]	Abnormal	-	N	-	-	Cont. ♀, > *p*95
110	MA 36-39	47,XY,+mar[26]/46,XY[12]	Identification (?)	N,N	N	-	-	Cont. ♂

Abbreviations: see Fig. 2 and Table 2.
[1] Previously described (in 't Veld et al., 1995b).

In one case (number 36) intra-uterine death (IUD) occured. Follow-up investigations and pregnancy outcomes in women with uncertainly abnormal karyotypes in STC-villi and a final normal (prenatal) cytogenetic diagnosis representing CPM (N=69) are presented in Table 3. In 62 cases a live infant was born and in three cases (numbers 62, 67 and 68) IUD occurred. TOP was carried out in four cases (numbers 78, 85, 88, and 90); in cases 88 and 90 because of ultrasound abnormalities, thought to be caused by the chromosomal abnormality, and in cases 78 and 85 before any follow-up investigation took place and after incorrect follow-up investigation, respectively.
Follow-up data of women with an undetermined prenatal cytogenetic diagnosis are shown in Table 4. TOP was carried out in two pregnancies; in case 107, it concerned selective termination of one fetus of a twin pregnancy at 12.5 weeks of gestation after confirmatory FISH investigation of an early amniotic fluid sample only.
Two pregnancies proceeded to term and resulted in the birth of normal babies. In case 106, IUD was established with ultrasound investigation after the findings of trisomy 13: fetal fibroblasts failed to grow following suction curettage

The final results of follow-up investigations of uncertainly abnormal karyotypes in STC-villi among the various indications, expressed as the confirmation rates [abnormal: (abnormal+ normal)] are presented in Table 5.

Table 5 - Results of follow-up investigations of uncertainly abnormal cytogenetic results in STC-villi among the various indications with calculated confirmation-rates [abnormal:(abnormal + normal)] or [generalized abnormalty:(generalized abnormality + CPM)]

		Final results			
Indication	Uncertainly abnormal results	Abnormal (generalized abnormality)	Normal (CPM)	Unknown (undetermined)	Confirmation rate (95% CI)
MA 36-39	78	24	50	4	0.324 (0.220 - 0.443)
MA ≥ 40	10	2	8	0	0.200 (0.025 - 0.556)
US ABN	9	6	2	1	0.750 (0.349 - 0.968)
CARRIER	0	0	0	0	-
DNA/BIO	3	0	3	0	0.000 (0.000 - 0.708)
OTHER	10	4	6	0	0.400 (0.122 - 0.738)
TOTAL	110	36	69	5	0.343 (0.252 - 0.434)

95% CI = 95% Confidence Interval

The confirmation rates of the different chromosomal abnormalities in STC-villi are shown in Table 6. The 219 abnormal karyotypes were non-mosaic in 147 (67%) and mosaic in 72 instances (33%). All 72 mosaic findings were uncertain; so, 65% of 110 uncertainly cytogenetic results concerned mosaic findings. Low, medium, and high level mosaicism was encountered in 30, 17, and 25 instances, respectively.

Table 6 - Generalized abnormalities versus CPM cases among the various initially uncertainly chromosomal abnormalities in STC-villi and the calculated confirmation-rates [generalized abnormality:(generalized abnormality + CPM)]

Chromosome abnormality	Uncertainly abnormal	Generalized abnormality	CPM	Undetermined	Confirmation rate (95% CI)
+13,+18	4	3	0	1	1.000 (0.292 -1.000)
+13,+18,+21 mosaicism					
low (≤ 10%)	7	0	7	0	0.000 (0.000 - 0.410)
medium (11-33.3%)	0	0	0	0	-
high (≥ 33.3%)	7	7	0	0	1.000 (0.590 - 1.000)
45,X	6	5	0	1	1.000 (0.478 - 1.000)
Sex chromosomal mosaicism					
low (≤ 10%)	16	0	15	1	0.000 (0.000 - 0.218)
medium (11-33.3%)	5	2[1]	3	0	0.400 (0.053 - 0.853)
high (≥ 33.3%)	3	2	1	0	0.667 (0.094 - 0.992)
Unusual trisomy (≠13,18,21)(mosaicism)					
full (100%)	10	4	6	0	0.400 (0.122 - 0.738)
low (≤ 10%)	4	0	4	0	0.000 (0.000 - 0.602)
medium	8	0	8	0	0.000 (0.000 - 0.369)
high (≥ 33.3%)(11-33.3%)	3	0	2	1	0.000 (0.000 - 0.708)
Structural rearrangements					
balanced	5	5	0	0	1.000 (0.478 - 1.000)
unbalanced	9	3	6[2]	0	0.333 (0.075 - 0.701)
Marker (mosaicism)					
full (100%)	1	1	0	0	-
low (≤ 10%)	2	0	2	0	0.000 (0.000 - 0.842)
medium (11-33.3%)	0	0	0	0	-
high (≥ 33.3%)	4	2	1	1	0.667 (0.094 - 0.992)
Mosaic structural rearrangements					
low (≤ 10%)	0	0	0	0	-
medium (11-33.3%)	2	0	2	0	0.000 (0.000 - 0.842)
high (≥ 33.3%)	5	0	5	0	0.000 (0.000 - 0.522)
Other (mosaicism)					
full (100%)	3	1	2	0	0.333 (0.084 - 0.906)
low (≤ 10%)	1	1	0	0	-
medium (11-33.3%)	2	0	2	0	0.000 (0.000 - 0.842)
high (≥ 33.3%)	3	0	3	0	0.000 (0.000 - 0.708)
All abnormalities					
full (100%)	38	22	14	2	0.611 (0.435 - 0.769)[3]
low (≤ 10%)	30	1	28	1	0.034 (0.001 - 0.178)[3]
medium (11-33.3%)	17	2	15	0	0.118 (0.015 - 0.364)[3]
high (≥ 33.3%)	25	11	12	2	0.478 (0.268 - 0.694)[3]
Total	110	36	69	5	0.343 (0.252 - 0.434)[3]

[1] Also high level mosaicism of Y-chromosomal structural abnormality (case 17, Table 2).
[2] Also low level sex chromosomal mosaicism (case 87, Table 3).
[3] Confirmation rates of the summarized non-mosaic and various mosaic results differ significantly ($(\chi^2 = 29.44$, $df = 3$, $p < 0.001$).

The majority of mosaic karyotypes turned out to be CPM (55/69 known cases, 80%), and the minority generalized mosaicism (14/69 known cases, 20%). Non-mosaic uncertainly cytogenetic results were more likely to be generalized mosaicism (22/36 known cases, 61%). The confirmation rates increased significantly in the rank order of low, medium, high level mosaicism and non-mosaicism (Table 6). The frequencies of generalized abnormalities among the various indications differed statistically significantly whilst those of CPM cases did not (Table 7). Hence, the predictive values of abnormal karyotypes in STC-villi [generalized abnormalities:(generalized abnormalities + CPM-cases)] in the various indications showed a statistically significant difference (Table 7).

Table 7 - Generalized abnormalities versus CPM-cases in STC-villi among the various indications and the calculated predictive values of abnormal cytogenetics [generalized abnormality: (generalized abnormality + CPM)] for the fetal karyotype

Indication	N	All abnormal	Generalized abnormality[1]	CPM[2]	Undetermined	Predictive value[3] (95% CI)
MA 36-39	2282	91 (4.0%)	37 (1.6%)	50 (2.2%)	4 (0.2%)	0.425 (0.320 - 0.536)
MA ≥ 40	447	23 (5.1%)	15 (3.4%)	8 (1.8%)	0 (0.0%)	0.652 (0.427 - 0.836)
US ABN	165	49 (29.7%)	46 (27.9%)	2 (1.2%)	1 (0.6%)	0.958 (0.858 - 0.995)
CARRIER	80	39 (48.8%)	39 (48.8%)	0 (0.0%)	0 (0.0%)	1.000 (0.910 - 1.000)
DNA/BIO	206	5 (2.4%)	2 (1.0%)	3 (1.5%)	0 (0.0%)	0.471 (0.230 - 0.722)
OTHER	319	12 (3.8%)	6 (1.9%)	6 (1.9%)	0 (0.0%)	
TOTAL	3499	219 (6.3%)	145 (4.1%)	69 (2.0%)	5 (0.1%)	0.678 (0.615 - 0.740)

[1] Frequencies of generalized abnormalities (US ABN and CARRIER combined) differ significantly (χ^2 = 593.16, df = 4, $p < 0.001$).
[2] Frequencies of CPM-cases (US ABN and CARRIER combined, DNA/BIO and OTHER combined) do not differ significantly (χ^2 = 2.45, df = 3, $p > 0.05$).
[3] Predictive values differ significantly (χ^2 = 64.68, df = 4, $p < 0.001$).

In a considerable number of patients (78/110, 71%), FISH on STC-villi slides was performed. In most instances (N=66) it concerned interphase FISH for the differentiation of coincidental local mitotic division errors from mosaicism in cases displaying mosaic trisomy, tetraploidy or sex chromosomal mosaicism. In 12 mosaic cases, 11 low level and one medium level mosaicism, FISH indicated a coincidental local mitotic division error, and follow-up amniocentesis was only performed in two instances. In 54 instances, FISH confirmed mosaicism in STC-villi; subsequent amniocentesis was carried out 48 times. In the remaining six cases, TOP was carried out (N=4), IUD occurred (N=1) or only follow-up ultrasound investigation could be performed (N=1). Amniocentesis was performed in 74 instances after uncertainly abnormal results in chorionic villi, and FISH on uncultured amniotic fluid cells for differentiation of generalized mosaicism from CPM in 55 cases. In 2 of these 55 cases

only FISH was performed and in 53 cases, FISH was followed by karyotyping cultured amniotic fluid cells. FISH was non-informative in one case (number 38 of Table 3) and FISH results and karyotypes showed a discrepancy twice; in case 14 of Table 2, FISH showed a normal signal distribution whereas the karyotype revealed a mosaic 45,X/46,XX, and in case 68 of Table 3, FISH with a 16-probe showed an increased percentage (26%) of three signals containing nuclei whereas the karyotype was normal. FISH was carried out in 12 cases on the metaphase level for identification of derivatives or marker chromosomes with various probes. In case 26 of Table 2, for instance, whole chromosome paint wcpX showed der(X) to stain completely; wcpY showed, in accordance with the information of the paint manufacturer (Cambio Ltd, Cambridge, U.K.), an aspecific signal on the expected place X(pter), but another, expected on X(q13), also on the p-arm of der(X), compatible with a pericentric inversion. Karyotyping and extended FISH-investigations on subsequently cultured amniotic fluid cells with the X-centromere probe (pBamX5) and the probes cpq23.1 (Xpter, Ypter), cAL24 (Xp21.2), and c7B2 (Xq28) confirmed the presence of a pericentric inversion.

All 12 live born infants in the generalized abnormality group (Table 2) were phenotypically normal. In the CPM group (Table 3), three of the 62 (5%) live born children had congenital malformations (Hirschsprung's disease in case 84, bilateral cleft in case 97, and hypospadia in case 100), and one displayed uniparental disomy 16 (maternal heterodisomy 16, case 66). The IUD cases (numbers 62, 67, and 68) showed multiple congenital malformations (MCA). Together with the two TOP cases (numbers 88 and 90) showing ultrasound malformations and another TOP case (no 78) showing MCA at autopsy, nine CPM cases (13.0%; 95% CI 6.1%-23.3%) displayed congenital malformations. The birthweights of children in the CPM group were located between the **p**5 and *p*95 in 54 of the 62 cases (87%). Three of the four children with birthweights exceeding the *p*95 were confined to the group of seven (mosaic) tetraploidy- CPM cases (P=0.05, binomial statistics).

Discussion

The composition of the group of abnormal cytogenetic results in STC-villi equals that of various previous studies (Ledbetter et al., 1992; Wolstenholme et al., 1994; Association of Clinical Cytogeneticists Working Party on Chorionic Villi in Prenatal Diagnosis, 1994; Pittalis et al., 1994). Our figure for CPM cases (69/3499; 2.0%) is perfectly comparable to that (101/4498; 2.2%) from Leschot et al. (1996). The proportion of generalized mosaicism of 20% among the mosaic results in STC-villi is somewhat higher than the reported figure of 10 - 12% (Ledbetter et al., 1992; Pittalis et al., 1994; Philips et al., 1996). The higher prevalence of generalized mosaicism in our series might be due to the fact that we had quite a number of high level mosaic trisomy 18 and 21 cases, all of which turned out to be generalized abnormal, although high level mosaicism of trisomy 18 is very unpredictable (Van Opstal et al., 1995).

Another factor might be that we consider the encounter of a "pseudomosaicism" in

amniotic fluid cells after the finding of mosaicism of the same abnormality in STC-villi, a generalized mosaicism rather than a combined pseudomosaicism and CPM as has been done by others (Ledbetter et al., 1992; Wolstenholme et al., 1994; Leschot et al., 1996). We found a statistically significant relation between the level of mosaicism in STC-villi and the likelihood of representing a generalized mosaicism. Although it is known that the distribution of normal and abnormal cells in mosaic placentae can be very irregular (Schuring-Blom et al., 1993; Henderson et al., 1996), the proportion of chromosomally abnormal cells in STC-villi seems to reflect the numbers of chromosomally abnormal and normal cells in the early cleavage stages and the chance of abnormal cells to be distributed to the fetal compartment as well. This issue has been profoundly discussed by Wolstenholme (1996), Robinson et al. (1997), and Los et al. (1998).

An increased risk of intra-uterine growth retardation (IUGR), fetal loss and poor pregnancy outcome has been reported in association with CPM (Schwinger et al., 1989; Johnson et al., 1990; Goldberg et al., 1990; Kalousek et al., 1991; Breed et al., 1991; Wapner et al., 1992; Brandenburg et al., 1996). The prevelance of 13% congenital malformations in our CPM group is in agreement with these reports. No increased frequency of IUGR was noted in our CPM cases, in agreement with the findings of Leschot et al. (1996). However, a statistically significant proportion of the children born after the prenatal diagnosis of mosaic tetraploidy-CPM showed birthweights above the *p*95; extreme birthweights, low as well as high, are to be expected in CPM cases (Wolstenholme et al., 1994).

Half of the abnormal karyotypes encountered in STC-villi turned out to be uncertain, and so, many prenatal follow-up investigations were required, amongst others 74 amniocenteses representing a second invasive procedure in 2.1% of the women undergoing chorionic villus sampling. These investigations were insufficient or incomplete in some of the 110 cases with uncertain results. This has led to two TOP procedures with the finding of normal karyotypes in fetal tissues. In two other cases normal karyotypes were also found in fetal fibroblasts after TOP, but in these cases the reasons for TOP were rather the severe ultrasound malformations. The investigation of the karyotype in LTC-villi as well might have resulted in continuation of the two, in hindsight, erroneously terminated pregnancies. The combined use of STC- and LTC-villi for the prenatal establishment of the fetal karyotype has been strongly advocated (Ledbetter et al., 1992; Pittalis et al., 1994; Hahnemann & Vejerslev, 1997a, b), but has in practice achieved in less than half of the cases (Ledbetter et al., 1992; Teshima et al., 1992). In our laboratory, the culturing of chorionic villi has never been a great success despite initially reported favourable results (Sachs et al., 1990). The limiting factor for successful culturing is the amount of villi obtained; it declined from a mean of 20 mg (Jahoda et al, 1990) to 15 mg in the years thereafter. At least 20 mg villus tissue is recommended for the successful performance of STC-villi and LTC-villi preparations (Smidt-Jensen et al., 1993); Leschot et al. (1996) stated that only cultures of chorionic villi were initiated in case of an amount of 35 mg villus tissue or more. We never used LTC-villi only as has been reported by others (Smidt-Jensen et al., 1993; Ledbetter et al., 1992; Teshima et al., 1992). In our opinion, the (partially avoidable) risk of a cytogenetic false-positive

misdiagnosis by using STC-villi alone (4/3499; 0.1%) was to be preferred over the unavoidable risk of 0.5 - 1.8% of maternal cell contamination potentially leading to diagnostic failures in LTC-villi (Ledbetter et al., 1992; Smidt-Jensen et al., 1993; Association of Clinical Cytogeneticists Working Party on Chorionic Villi in Prenatal Diagnosis, 1994; Saura et al., 1997). Saura et al. (1997) reported that metaphases were completely of maternal origin in 0.5% of cultured chorionic villi. Concerning false-negative results, LTC-villi are more reliable than STC-villi (Ledbetter et al., 1992; Pittalis et al., 1994; Hahnemann & Vejerslev, 1997a), especially in cases with a high risk for cytogenetic abnormalities (Kennerknecht et al., 1993). Fortunately, we did not encounter noticeable false-negative results in our study period. For the quality of the karyotype, LTC-villi are preferable over STC-villi. Finally, for the discovery of potential cases of fetal UPD, LTC-villi alone are not suitable, since UPD cases are only associated with CPM types I and III (Wolstenholme, 1996; Los et al., 1998).

FISH on STC-villi preparations and on uncultured amniotic fluid cells turned out to be effective for the differentiation of coincidental local mitotic division errors from mosaicism in STC-villi, and generalized mosaicism from CPM, respectively. A remarkable discrepancy was encountered in case 68 of Table 3, which has already been discussed in detail (Van Opstal et al., 1998).

The certainty rates and predictive values of abnormal cytogenetic results of STC-villi in the various indications showed that the cytogenetic performance is only acceptable when the cytogenetic risk is substantially increased. The best results were achieved in women with the highest risks: carriers of structural rearrangements and women displaying fetal abnormalities on ultrasound. Therefore, we advise chorionic villus sampling to those women with singleton pregnancies and a cytogenetic risk equal to or greater than that of a 40-year-old pregnant woman, averaging 3.4% in the first trimester of pregnancy for all abnormalities (Hook, 1990), or 2.7% for just the trisomies 13, 18 or 21 (Snijders et al., 1994). In this limited group of women we prefer chorionic villus sampling over amniocentesis. In twin or triplet pregnancies, we prefer chorionic villus sampling allowing for a selective TOP as early as possible in case of discrepant results (Brambati et al., 1996); our case 107 of Table 3 is an example of this policy. We also prefer chorionic villus sampling in the case of prenatal DNA or biochemical investigations representing a high genetic risk in most cases and in which cytogenetics is only additional. Finally, some "young" women (of advanced maternal age) will of course insist on chorionic villus sampling as will some "older" women insist on amniocentesis, and their wishes should be respected.

In conclusion, prenatal cytogenetic diagnosis on STC-villi alone required a high number of prenatal follow-up investigations. Notwithstanding this fact, there remained a risk for false-positive results. Cytogenetics should preferentially be carried out on STC-villi and LTC-villi simultaneously in order to improve the quality of the karyotype, to reduce the risk of false-positive results and to eliminate the theoretical risk of false-negative diagnoses. For this reason we improved the yield of obtained tissue in chorionic villus sampling, reintroduced the culturing of villi and introduced the routine analysis of LTC-villi additional to STC-villi in 1997 in our department. We prefer chorionic villus sampling over amniocentesis on cytogenetic indications only when a cytogenetic risk of at least 3% is present.

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Chapter 3

Cytogenetic investigation in STC-villi and LTC-villi

ACCURACY OF ABNORMAL KARYOTYPES AFTER THE ANALYSIS OF BOTH SHORT- AND LONG-TERM CULTURE OF CHORIONIC VILLI

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Summary

We report in detail the cytogenetic results of 1838 consecutive chorionic villus samples with the availability of both short-term culture (STC-villi) and long-term culture (LTC-villi) preparations in 1561 cases (84.9%).
A high degree of laboratory success (99.5%) and diagnostic accuracy (99.8%) was observed; in four cases of low mosaicism, all four associated with the final birth of a normal child, a small risk of uncertainty was accepted. The combined analysis of STC- and LTC-villi reduced follow-up amniocenteses by one-third in comparison with the analysis of STC-villi alone. We believe that the desired level of quality and accuracy of prenatal cytogenetics in chorionic villi can only be achieved when both STC- and LTC-villi are available. We conclude that CVS might then be the mode of prenatal diagnosis of first choice in pregnancies with a high (cytogenetic) risk.

Introduction

Since its introduction in our department in 1983, first-trimester chorionic villus sampling has become a welcome alternative to mid-trimester amniocentesis, especially for women with a high genetic risk (Los et al., 1998; Sachs et al.,1988). However, the safety of chorionic villus sampling as well as the accuracy of cytogenetic laboratory findings in chorionic villi (CV) have been subject to intensive investigations (Brambati et al., 1990, 1991; Jackson et al., 1992; Smidt-Jensen et al., 1992; Hahnemann and Vejerslev, 1997a; Smith et al., 1999; WHO/PAHO Consultation on CVS, 1999).

The prevalent problem in CV is the occurrence of chromosomal mosaicism in 1-3% of CV cases (Kalousek et al., 1992). Both true mosaicism and confined placental mosaicism (CPM) are well documented (Kalousek and Dill 1983; Wang et al., 1994; Kalousek and Vekemans, 1996). The three conditions of an abnormal karyotype in 'semi-direct' preparations (short-term cultured villi; STC-villi), in long-term cultured villi (LTC-villi) and in both STC- and LTC-villi combined with a normal karyotype in the fetus (or amniotic fluid cells) are defined as CPM type I, II, and III, respectively (Kalousek and Barrett, 1994; Kalousek and Vekemans, 1996; Wolstenholme et al., 1996). True mosaicism can be generalised mosaicism with absolute or relative concordance (GMAC, or GMRC, respectively). In the case of GMAC, mosaicism is encountered in all three compartments (STC-villi, LTC-villi and fetus proper) whilst in the case of GMRC, mosaicism in at least one compartment is accompanied by the non-mosaic abnormality in the other compartment(s). Additionally, generalized mosaicism with a discrepant (normal) karyotype in STC-villi (GMDD) or LTC-villi (GMDC), can be distinguised. Finally, the finding of different abnormal karyotypes in all three compartments is known as generalised mosaicism with total discrepancy (GMTD) (Pittalis et al., 1994).

Several studies (Ledbetter et al., 1992; ACC, 1994; Pittalis et al., 1994; Hahnemeann and Vejerslev, 1997a; Los et al., 1998) have shown that the results of STC-villi are less representative for the fetal status than those of LTC-villi or the combined results of both. The analysis of STC-villi only may result in an inappropriate number of follow-up amniocenteses and even unnecessary termination of pregnancy in some cases (Los et al., 1998). Combining the cytogenetic analysis of both STC- and LTC-villi is gaining popularity among several laboratories, and experiences with large series have become available (Smidt-Jensen et al., 1993, Pittalis et al., 1994; ACC, 1994; Smith et al.,1999). To minimize diagnostic pitfalls and interpretation dilemmas of mosaic findings, the routine analysis of both STC- and LTC-villi was introduced into our department in 1997 (Los et al., 1998; Van den Berg et al., 1999).

During a 3-year period we focussed our attention to the combined cytogenetic results of STC- and LTC- villi. The aims of this study were:

1. To establish the improvement of chromosome quality by the investigation of LTC-villi.
2. To investigate the reliability of cytogenetic results in both villi compartments.
3. To compare the reduction-rate in the number of follow-up amniocenteses with the figure from our previous study with the analysis of STC-villi alone.

Materials and Methods

During a 3-year period a total of 2032 chorionic villus samples were received in our department. Chorionic villus samples were obtained under continuous ultrasound guidance by transabdominal aspiration as described previously (Jahoda et al., 1990). At least 20 mg of villi are required for chromosomal analysis of both STC- and LTC-villi in those cases where the indication for prenatal diagnosis is cytogenetic only. In cases where primarily DNA or biochemical investigation is indicated, at least 40 mg of villi are required when additional chromosomal analysis is also requested.

Cytogenetic investigation was performed in 1838 cases. The indications for cytogenetic investigation included advanced maternal age (MA)(≥36 years), the parents being carriers for structural rearrangements or marker chromosomes (Carrier), ultrasound abnormalities (US Abn), recurrence risk of chromosomal abnormalities, a previous child with multiple congenital abnormalities, or a family history of chromosomal abnormality, and other reasons (Other) (n = 1663). In the remaining 175 cases the reason for prenatal diagnosis was a recurrence risk for Mendelian inherited diseases detectable with DNA analysis or various metabolic diseases (DNA/BIO); secondary chromosomal analyses were performed.

The aspirated villi samples were carefully washed and estimated under an inverted microscope. Villi fragments were isolated from maternal tissue and blood clots. For the STC-villi preparation 15 mg were incubated overnight using fluorodeoxyuridine (FdU) synchronization (Gibas et al., 1987). The remaining villi were simultaneously used for the preparation of LTC-villi, which consisted of a trypsin - EDTA and collagenase treatment at 37°C for 60 and 120 minutes, respectively (Smidt-Jensen et al., 1989). Cells were cultured in Lab-tek®II Chamber slides™ (Nalge Nunc International, Naperville, IL, USA) and harvested *in situ* after 5-7 days. Karyotyping was routinely performed by G-banding using thePancreatin-Trypsin-Giemsa staining technique. Sometimes a DA-DAPI staining was used in addition. In one case R-banding was used for inactivation studies of a balanced (X;autosome) translocation using methotrexate cell synchronisation and bromodeoxyuridine incorporation according to standard protocols. The inactivation pattern was studied in LTC-villi, amniotic fluid (AF) cells and fetal blood (FB) lymphocytes.

The numbers of metaphases that were investigated in STC- and LTC-villi under various circumstances are presented in Table 1 and Figure 1. In cases where LTC-villi were non-available, normally 16 cells were analysed in STC-villi as described previously (Los et al., 1998). A karyotype in STC- and/or LTC-villi was considered normal in cases of 46,XX or 46,XY ± inversion (9)(p12q13) and/or ± any one-cell abnormality. All other results were considered abnormal. These could either be non-mosaic in STC- and LTC-villi (all cells in both compartments abnormal) or mosaic in STC- and/or LTC-villi (two or more cells abnormal in one or two compartments). Previously, abnormal karyotypes in STC-villi have been divided into certainly abnormal (abnormal) and uncertainly abnormal (uncertain) results (Los et al., 1998).

Table 1 -The number of metaphases investigated in various circumstances in STC-and LTC-villi

		STC-villi (n)	LTC-villi (n)
Normal karyotype in STC-villi		8	8
Numerical aberration in first 8 STC-villi cells			
+21		16	-
Triploidy		12	-
47,XXY 47,XXX 47,XYY		16	8
+13, +18			
with US abnormality		16	-
without US abnormality		16	8
45,X			
with US abnormality		16	8
without US abnormality		16[a]	8[a]
Aneuploidy ≠13, 18, 21, X and Y		16	8
Mosaic aneuploidy		20[a]	10[a]
One cell abnormality +7,+8,+9,+11,+13,+14,+15,+18,+21,+mar		20	10
Tetraploidy		special protocol (Figure 1)	
Other		consideration in each case	
Structural rearrangement in first 8 STC-villi cells			
Familiar balanced		8	8
Familiar unbalanced		16	-
De -novo	100 %	16	8
	mosaic	20	10

[a] = Additional FISH investigations on interphase nuclei.

In the present study, abnormal karyotypes are divided into six categories:

1. Abnormal STC-villi which were assumed to represent generalized abnormalities and allowed a clinical decision without further prenatal investigations.
2. Uncertain STC-villi in combination with normal LTC-villi, giving a certainly normal cytogenetic result (abnormalities confined to the STC-villi; CPM type I).
3. Uncertain STC-villi in combination with abnormal LTC-villi, giving a certainly abnormal cytogenetic result (generalized abnormalities).
4. Uncertain STC-villi with normal or abnormal LTC-villi, giving an uncertain combination of cytogenetic results requiring follow-up investigation.
5. Normal STC-villi with abnormal LTC-villi, giving an uncertain combination of cytogenetic results requiring follow-up investigation.
6. Uncertain STC-villi, LTC- villi non-available, follow-up investigations indicated.

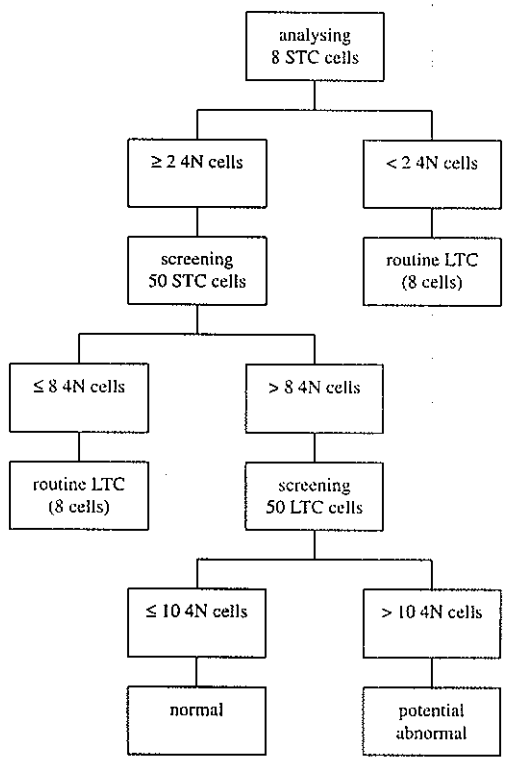

Figure 1 - Management plan for the finding of tetraploid cells in STC- and LTC-villi, according to Noomen et al. (2001). 4N cells = tetraploid cells; 2N cells = diploid cells.

In several cases additional FISH on interphase nuclei (n=200) of STC- and LTC-villi was performed in cases with aneuploidy mosaicism for the differentiation between a coincidental local mitotic division error and mosaicism or for a more accurate establishment of the distribution of abnormal cells in both villi compartments (Van den Berg et al., 1997; Los et al., 1998; Van Opstal et al., 1998). In some cases FISH on metaphases of STC- or LTC-villi was carried out for the verification of small reciprocal translocations and marker chromosomes. In cases of structural rearrangements or mosaicism of a marker chromosome, parental karyotypes were prepared according to standard techniques.

Follow-up investigation involved amniocentesis with FISH on (uncultured) amniotic fluid cells, regularly followed by chromosome analysis on cultured amniotic fluid cells, DNA studies and/or ultrasound investigation. FISH on uncultured amniotic fluid cells (n=100) was performed for the differentiation between CPM and generalized mosaicism. For these investigations the same probes

were used in STC-, LTC-villi and amniocytes (Van den Berg et al., 1997; Van Opstal et al., 1998). Amniotic fluid cells were cultured in Lab-tek®II Chamber slides™ according to standard protocols.

In some cases DNA studies were performed for the investigation of maternal cell contamination, cross contamination, uniparental disomy (UPD) or the parental origin of a supernumerary chromosome (Van den Berg et al., 1997; Van Opstal et al., 1998).

Follow-up ultrasound investigations were performed in our department as described previously (Wladimiroff et al., 1995). In cases of termination of pregnancy (TOP), confirmation of the cytogenetic abnormality was performed in as many cases as possible.

Information about the pregnancy outcome of all CPM cases and translocation carriers was received from the women, the referring midwives or physicians.
Statistical analysis comprised χ^2 tests (of proportions).

Results

Cytogenetic investigations were performed in 1838 chorionic villus samples (Table 2). STC-villi and LTC-villi preparations could be made of 1561 samples. In 277 cases LTC-villi were non-available. In 1829 cases (99.5%) a cytogenetic result was achieved. In nine cases, no cytogenetic diagnosis could be made due to laboratory failure in eight cases and insufficient material in one case, respectively. The failure-rate of LTC-villi was 22/1561 (1.4%). Normal karyotypes in STC- and/or LTC-villi preparations were found in 1671 samples (91.4%). An example of karyotypes in STC- and LTC-villi preparations of the same sample is shown in Figure 2.

Table 2 -Total number of chorionic villus samples received during the 3-year period (1997-1999)

	Total		1997		1998		1999	
Total CVS	2032		670		706		656	
DNA/BIO investigation only	194		57		68		69	
Cytogenetic investigation	**1838**		**613**		**638**		**587**	
STC+LTC investigation	**1561**		**400**		**610**		**551**	
STC+LTC results	1463	93.7 %	366	91.5 %	579	94.9 %	518	94.1 %
STC results (LTC not analysed)	72	4.6 %	20	5 %	29	4.8 %	23	4.2 %
STC results (LTC failure)	22	1.4 %	14	3.5 %	2	0.6 %	6	0.4 %
LTC results (STC failure)	4	0.3 %	0		0		4	1.4 %
STC investigation (LTC non-available)	**277**		**213**		**28**		**36**	
STC results	268	96.8 %	205	96.2 %	28	100 %	35	97.2 %
STC failure	9	3.2 %	8	3.8 %	0		1[a]	0.8 %
Total cytogenetic results	**1829**	99.5 %	**605**	98.7 %	**638**	100 %	**586**	99.8 %

a = <<5mg

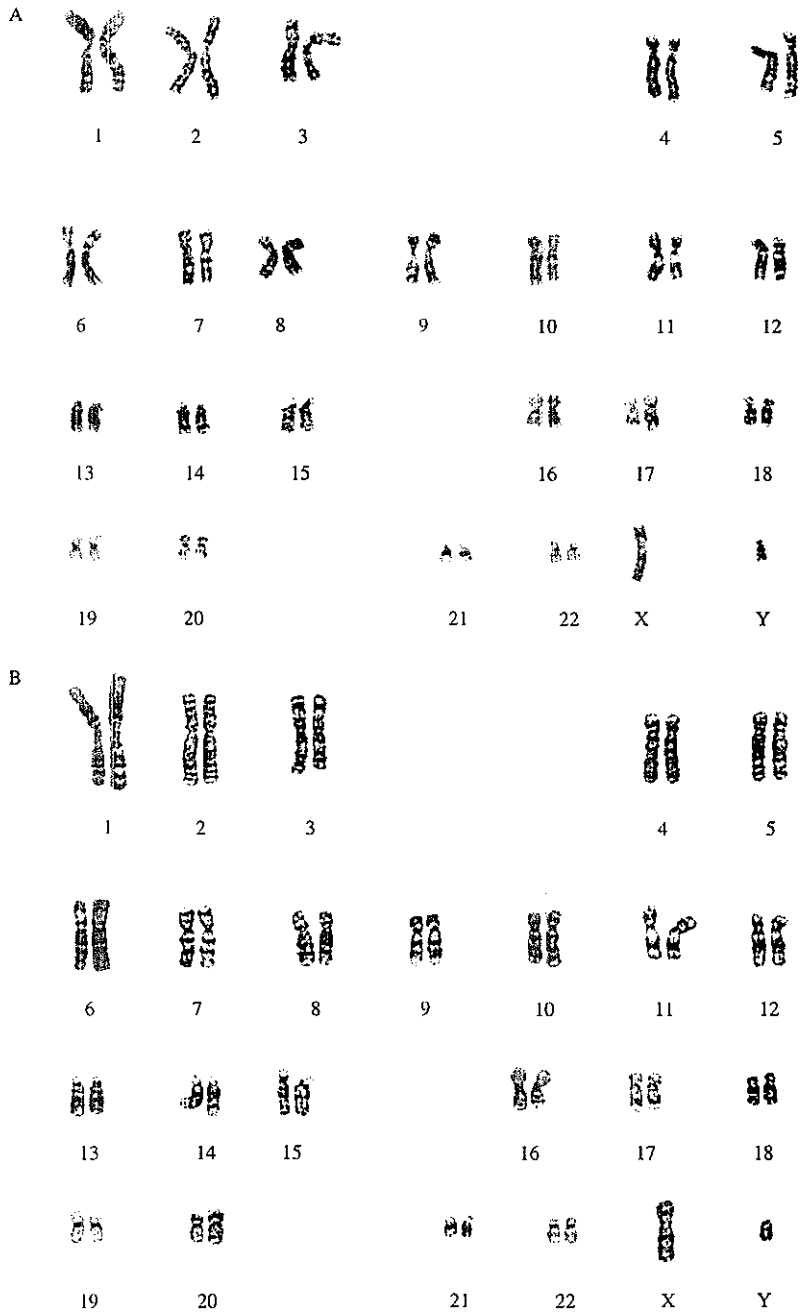

Figure 2 - Karyotypes of one CV sample after **(A)** STC-villi preparation showing about 300 bands and **(B)** LTC-villi preparation showing about 400 bands

The frequency of maternal cell contamination (MCC) in LTC-villi was very low. MCC has occurred in only three cases among 761 identified male cases (0.4%). The MCC in those cases did not lead to diagnostic errors. Abnormal karyotypes, in STC-villi and/or LTC-villi preparations were seen in 158 cases (8.6%). These 158 cases fitted into our six categories of certain and uncertain karyotypic combinations in the STC- and LTC-villi preparations. The exact numbers and the distribution among the six categories in relation to the nature of the various abnormalities are shown in Table 3.

Ninety-one of these abnormal karyotypes were considered certainly abnormal after the analysis of STC-villi preparations only. In the other 67 cases an uncertain result was achieved in either STC-, LTC-villi or both preparations. Detailed information about these 67 cases among the various categories, is presented in the Tables 4-8.

Table 4 summarizes the different karyotypes, further investigations, as well as the cytogenetic interpretation and pregnancy outcome in women with uncertain karyotypes in STC-villi and a normal karyotype in LTC-villi, and finally normal cytogenetic results. All cases turned out to be CPM type I.

Table 5 summarizes the different karyotypes, additional and follow-up investigations, the cytogenetic interpretation and pregnancy outcome in women with finally abnormal cytogenetic results (11 abnormalities, 9 carriers). TOP was carried out in ten pregnancies. The cytogenetic abnormality was confirmed in nine cases, in one case fibroblasts failed to grow. One pregnancy (No. 18) continued at the parents' request. Follow-up amniocentesis was only performed in two instances; in one instance at the explicit request of the parents (No. 19) and revealed the same chromosome aberration. TOP was requested and carried out. In the other case (No. 14) subsequent amniocentesis and cordocentesis were carried out simultaneously for X-inactivation studies. These studies conducted on LTC-villi, AF and FB cells showed the normal X to be inactive and the derivative X chromosome (and the derivative 9) to be active in all investigated cells (50 metaphases per sample).

Table 6 summarizes the karyotypes, additional and follow-up investigations, cytogenetic interpretation and pregnancy outcome of uncertain STC-villi with normal or abnormal LTC-villi resulting in a finally uncertain cytogenetic result. There were six cases of CPM type I, two cases of CPM type III, one carrier and two instances of sampling problems leading to mosaic karyotypes. In five cases (Nos 1-5) FISH was performed on interphase level for the demonstration or exclusion of a mosaicism in either STC- or LTC-villi or for the establishment of a more accurate level of mosaicism in STC-villi. In case No.1, subsequent amniocentesis was carried out for the differentiation of CPM I from GMDC. FISH on uncultured amniocytes revealed a normal signal distribution with a chromosome 13 specific probe and therefore the diagnosis of a rare CPM type I of non-mosaic trisomy 13 could be made. The pregnancy continued and resulted in the birth of a healthy male infant.

In case No. 3 the possibility of a real sex chromosomal mosaicism was considered and subsequent amniocentesis was carried out; FISH on uncultured amniocytes revealed a normal XY signal distribution (Van den Berg et al., 2000).

Table 3 - Karyotypes in STC-, STC- and LTC-villi of women undergoing chorionic villus sampling during the period 1997 - 1999

	STC abnormal			Combination STC + LTC certain						Combination STC + LTC uncertain								
				STC uncertain LTC normal			STC uncertain LTC abnormal			STC uncertain LTC normal/abnormal			STC normal LTC abnormal			STC uncertain LTC not available		
	'97	'98	'99	'97	'98	'99	'97	'98	'99	'97	'98	'99	'97	'98	'99	'97	'98	'99
Trisomy 13,18	4	11	4	-	-	-	2	2	-	-	1	-	-	-	-	-	-	-
Trisomy 21	13	11	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Triploidy	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sex chromosomal aneuploidy																		
100%	3	3	7	-	-	-	1	-	2	-	1	1	-	-	-	-	1	-
mosaic	-	-	-	1	2	2	-	-	-	2	-	1	1	-	-	1	-	1
Marker chromosomes																		
100%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
mosaic	-	-	-	2	-	1	1	1	-	-	-	1	1	1	-	1	-	1
Structural rearrangements																		
balanced	9	7	5	-	-	-	1	2	5	-	-	-	-	-	-	1	-	-
unbalanced	-	2	-	-	-	-	-	-	1	1	1	-	-	-	-	1	-	-
Unusual trisomy ≠ 13,18,21	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
Autosomal mosaic 13,18,21	1	1	-	-	-	-	-	-	-	-	-	-	-	-	3	1	-	-
Autosomal mosaic ≠ 13,18,21	-	-	-	2	1	1	-	-	-	-	-	-	1	5	-	-	-	1
Mosaic structural rearrangements	-	-	-	-	-	3	-	2	-	-	-	2	-	-	-	-	-	-
Total number	30	36	25	5	3	5	5	7	8	3	3	5	4	6	4	5	1	3
Parental karyotype	-	1	-	-	-	-	2	4	6	1	1	2	-	-	-	3	-	-
FISH investigation	3	-	-	3	3	3	1	5	3	3	3	4	3	4	5	1	-	2
Amniocenteses	-	-	-	-	-	-	-	2[a]	-	3	2	2[b]	2	3	4	4	1	1

a = One amniocentesis at explicit request of the parents, one for X inactivation study.
b = One amniocentesis for UPD investigation.
Certain karyotypes appear unshaded in the table, whereas uncertain karyotypes are indicated by grey shading

Table 4 - Uncertain STC-villi karyotypes with normal LTC-villi karyotypes finally resulting in normal cytogenetic results

Case	Indication	STC-villi Karyotype	STC-villi FISH	LTC-villi Karyotype	LTC-villi FISH	Parental karyotype	US	Amniocentesis Karyotype	Amniocentesis FISH	Cytogenetic Interpretation & Pregnancy outcome
1	MA 36-39	45,X[2]/46,XY[18]	N	46,XY	N	-	-	-		CPM I Cont. healthy ♂
2	Other	45,X[2]/46,XY[21]	N	46,XY	N	-	-	-	-	CPM I Cont. healthy ♂
3	MA 36-39	92,XXYY[3]/45,X[2]/ 46,XY[47]	N	46,XY	-	-	-	-	-	CPM I Cont. healthy ♂
4	US Abn	45,X[2]/46,XX[18]	A	46,XX[10] [b]	N	-	-	-	-	CPM I TOP -
5	MA 36-39	45,X[3]/46,XX[17]	A	46,XX[10] [b]	N	-	-	-	-	CPM I Cont. healthy ♀
6[a]	Other	47,XY,+mar[21]/46,XY[5]	-	46,XY[24]	-	-	-	-	-	CPM I Cont. healthy ♂
7	MA 36-39	47,XY,+mar[2]/46,XY[17]	-	46,XY[16]	-	-	-	-	-	CPM I Cont. healthy ♂
8	MA 36-39	47,XX,+7[3]/46,XX[17]	A	46,XX	N	-	-	-	-	CPM I Cont. healthy ♀
9	MA 36-39	47,XX,+7[2]/46,XX[19]	N	46,XX	N	-	-	-	-	CPM I Cont. healthy ♀
10[a]	MA 36-39	47,XX,+22[4]/46,XX[16]	A	46,XX	N	-	-	-	-	CPM I Cont. healthy ♀
11	Carrier	50,XX,+3,+5,+7,+8[5]/ 46,XX[15]	-	46,XX	-	-	-	-	-	CPM I Cont. healthy ♀
12	Other	46,XX,add(21)(p?)[5]/ 46,XX[15]	N	46,XX	-	-	-	-	-	CPM I Cont. healthy ♀
13	MA 36-39	46,X,t(Y;15)[27]/46,XY[19]	-	46,XY[25]	-	-	-	-	-	CPM I Cont. on going

a = One of twin pregnancy, the other twin displayed a normal karyotype.
b = Maternal cell contamination not excluded.
A = Abnormal; add = additional material of unknown origin; Cont. = continuation of pregnancy; I = identification; mar = marker chromosome; N = normal; TOP = termination of pregnancy; US = follow-up ultrasound investigation.

Table 5 - Uncertain STC-villi karyotypes with abnormal LTC-villi karyotypes finally resulting in abnormal cytogenetic results

Case	Indication	STC-villi Karyotype	STC-villi FISH	LTC-villi Karyotype	LTC-villi FISH	Parental karyotype	US	Amniocentesis Karyotype	Amniocentesis FISH	Cytogenetic Interpretation & Pregnancy outcome
1	MA ≥40	47,XX,+18	-	47,XX,+18	-	-	A	-	-	A TOP confirmed
2	MA ≥40	47,XY,+18	-	47,XY,+18	-	-	A	-	-	A TOP confirmed
3	MA 36-39	47,XX,+18	-	47,XX,+18	-	-	N	-	-	A TOP confirmed
4	US Abn	47,X,del(Y),+18[16]/ 46,X,-Y,+18[14]	A	47,X,del(Y),+18[7]/ 46,X,-Y,+18[1]	-	N/N	-	-	-	A TOP confirmed
5	MA 36-39	45,X	-	45,X	-	-	A	-	-	A TOP confirmed
6	MA 36-39	45,X	-	45,X	-	-	N	-	-	A TOP confirmed
7	MA 36-39	47,XXY	-	47,XXY	-	-	-	-	-	A TOP confirmed
8	MA 36-39	47,XX,+mar[28]/46,XX[2]	I (?)	47,XX,+mar[24]	-	N/N	-	-	-	A TOP -
9	MA 36-39	47,XX,+mar[16]/46,XX[2]	I (?)	47,XX,+mar[10]/46,XX[8]	-	mar pat	-	-	-	Carrier Cont. healthy ♀
10	MA 36-39	46,XY,t(7;11)	-	46,XY,t(7;11)	-	t(7;11)mat	-	-	-	Carrier Cont. healthy ♂
11[a]	MA 36-39	45,XY,der(13;14)	-	45,XY, der(13;14)	-	N/N	-	-	-	Carrier Cont. 22 wks ppt
12	Other	45,XX,der(13;14)	-	45,XX,der(13;14)	-	t(13;14)pat	-	-	-	Carrier Cont. healthy ♀
13	US Abn	45,XX,der(13;14)	-	45,XX,der(13;14)	-	t(13;14)mat	-	-	-	Carrier Cont. healthy ♀
14	Carrier[b]	46,X,t(X;9)	I (A)	46,X,t(X;9)	-	-	N	46,X,t(X;9)	-	Carrier Cont. healthy ♀
15[a]	MA 36-39	46,XX,t(18;19)	-	46,XX,t(18;19)	I (A)	N/N	N	-	-	Carrier Cont. healthy ♀
16	MA ≥40	46,XY,t(10;11)	I (A)	46,XY,t(10;11)	-	N/N	-	-	-	Carrier Cont. healthy ♂
17	MA 36-39	46,XX,der(21)	I (N)	46,XX,der(21)	N	v(21)pat	-	-	-	Carrier Cont. on going
18	US Abn	46,XX,der(21)	-	46,XX, der(21)	-	t(9;21)pat	-	-	-	A Cont. ♀ MCA
19	Carrier[c]	45,X,t(19;21)[16]/ 46,X,der(Y),t(19;21)[4]	A	45,X,t(19;21)[13]/ 46,X,der(Y),t(19;21)[20]	A	-	-	45,X,t(19;21)[42] 46,X,der(Y), t(19;21)[9]	A	A TOP confirmed
20	MA 36-39	47,XY,+idic(15)[12]/ 46,XY[6]	I (A)	47,XY,+idic(15)[12]/ 46,XY[5]	I (A)	N/ -	-	-	-	A TOP confirmed

a = One of twin pregnancy, the other twin displayed a normal karyotype; b = Maternal carriership t(13;14).; c = Maternal carriership t(19;21); ? = not identified; der = derivative; idic = isodicentric chromosome; mat = maternal; pat = paternal; MCA = multiple congenital malformations. For. abbreviation see also Table 4.

Table 6 - Uncertain STC-villi with normal or abnormal LTC-villi karyotypes finally resulting in uncertain cytogenetic results

Case	Indication	STC-villi Karyotype	STC-villi FISH	LTC-villi Karyotype	LTC-villi FISH	Parental karyotype	US	Amniocentesis Karyotype	Amniocentesis FISH	Cytogenetic Interpretation & Pregnancy outcome
1	MA 36-39	47,XY,+13	A	46,XY	N	-	-	-	N	CPM I Cont. healthy ♂
2	MA 36-39	47,XXX	A	46,XX [c]	N	-	-	46,XX	N	CPM I Cont. healthy ♀
3	MA 36-39	45,X	A	46,XY	N	-	-	46,XY	N	CPM I Cont. healthy ♂
4[b]	MA ≥40	45,X[28]/46,XX[22]	A	46,XX [c]	N	-	-	46,XX	N	CPM I Cont. healthy ♀
5[b]	MA ≥40	45,X[28]/46,XX[2]	A	no metaphases	A	-	-	46,XY	N	wrong sampled Cont. healthy ♂
6[a]	MA 36-39	46,XX[21]/46,XY[10]	-	46,XX[10]/46,XY[20]	-	-	-	-	-	cross cont. Cont. healthy ♀ and ♂
7	US Abn	46,XY,del(2)(p?)	I (A)[d]	46,XY,del(2)(p?)	-	t(1;2)mat	-	-	-	Carrier TOP ♂ MCA
8	MA 36-39	46,XY,der(3)(q?)	I (N)	46,XY,der(3)(q?)	-	N/N	-	46,XY	-	CPM III Cont. healthy ♂
9	US Abn	46,XY,der(16?)[5]/46,XY[3]	I (N)	46,XY,der(16?)[1]/ 46,XY[6]	I	-	-	-	-	CPM III TOP 46,XY
10	MA ≥40	47,XY,+mar[12]/46,XY[9]	I (A)	47,XY,+mar[1]/46,XY[29]	N	N/N	N	-	-	CPM I Cont. healthy ♂
11	MA ≥40	46,XY,der(15)[20]/46,XY[10]	I (A)	46,XY	N	N/N	-	46,XY	N	CPM I Cont. healthy ♂

a = One of twin pregnancy, the other twin displayed a normal karyotype.
b = Twin pregnancy; previously described (Van den Berg et al.,1999).
c = Maternal cell contamination not excluded.
d = FISH revealed a t(1;2), del = deletion.
For abbreviation see also Table 4 and 5.

Table 7 - Normal STC-villi with abnormal LTC-villi karyotypes finally resulting in uncertain cytogenetic results

Case	Indication	STC-villi Karyotype	STC-villi FISH	LTC-villi Karyotype	LTC-villi FISH	Parental karyotype	US	Amniocentesis Karyotype	Amniocentesis FISH	Cytogenetic Interpretation & Pregnancy outcome
1	MA 36-39	46,XX	N	47,XXX[3]/46,XX[15]	-	-	-	46,XX	N	CPM II Cont. healthy ♀
2	MA ≥40	46,XY	N	47,XY,+ i(21)(q10)	A	-	-	46,XY	N	CPM II Cont. on going
3	MA ≥40	46,XY	-	47,XY,+mar[2]/46,XY[28]	-	-	-	-	-	CPM II? Cont. healthy ♂
4[a]	MA ≥40	46,XX	-	47,XX,+mar[2]/46,XX[25]	-	-	-	-	-	CPM II? Cont. healthy ♀
5	carrier	45,XX,der(13;14)	N	46,XX,+8,t(13;14)	A	-	N	-	N	CPM II Cont. healthy ♀
6	MA 36-39	46,XX	N	47,XX,+18[10]/46,XX[20]	A	-	-	46,XX	N	CPM II Cont. healthy ♀
7	carrier	45,XY,der(13;14)	N	46,XY,der(13;14),+18 [4]/ 45,XY,der(13;14)[28]	A	-	-	45,XY,der(13;14)	N	CPM II Cont. healthy ♂
8	MA ≥40	46,XY	N	47,XY,+21[19]/46,XY[4]	A	-	-	-	N	CPM II Cont. healthy ♂
9	MA 36-39	46,XX	N	47,XX,+2[2]/46,XX[14]	A	-	N	Recommended	-	CPM II? Cont. healthy ♀
10	US Abn	46,XY	-	47,XY,+8[2]/46,XY[18]	N	-	N	-	-	CPM II Cont. healthy ♂
11	DNA/BIO	46,XY	N	47,XY,+9[2]/46,XY[17]/ 46,XX[6]	A	-	-	46,XY	N	CPM II Cont. - MCC
12	MA 36-39	46,XX	N	47,XX,+10[12]/46,XX[7]	A	-	-	-	N	CPM II Cont. healthy ♀
13	MA 36-39	46,XX	N	47,XX,+20[2]/46,XX[14]	N	-	-	-	-	CPM II Cont. healthy ♂
14	MA ≥40	46,XY	A	47,XY,+16[7]/46,XY[9]	A	-	-	-	N	CPM III Cont. healthy ♂

a = One of twin pregnancy, the other twin displayed a normal karyotype.
For abbreviation see also Table 4 and 5.

Table 8 - Uncertain STC-villi , LTC-villi non-available

Case	Indication	STC-villi Karyotype	STC-villi FISH	LTC-villi Karyotype	LTC-villi FISH	Parental karyotype	US	Amniocentesis Karyotype	Amniocentesis FISH	Cytogenetic Interpretation & Pregnancy outcome		
1	MA ≥40	47,XXY	-	-	-	-	-	47,XXY	A	A	Cont.	healthy ♂
2[a]	MA 36-39	45,X[6]/46,XY[24]	-	-	-	-	N	46,XY	N	CPM I or III	Cont.	healthy ♂
3[a]	MA 36-39	45,X[4]/46,XX[26]	A	-	-	-	-	-	-	CPM I or III	Cont.	healthy ♀ 46,XX
4	DNA/BIO	46,X,+mar[13]/45,X[5]/ 46,XX[12]	A	-	-	-	-	46,XX	N	CPM I or III	Cont.	healthy ♀
5	MA ≥40	47,XX,+mar[15]/46,XX[28]	-	-	-	N/N	N	46,XX	-	CPM I or III	Cont.	healhty ♀
6	MA 36-39	46,XX,inv(3)	-	-	-	inv(3)pat	-	-	-	carrier	Cont	healthy ♀
7	Other	46,XY,del(22)(q?)	-	-	-	t(11;22)mat	-	46,XY,t(11;22)	-	carrier	Cont.	healthy ♂
8	MA 36-39	47,XY,+18[9]/46,XY[22]	A	-	-	-	N	46,XY	N	CPM I or III	Cont.	healthy ♂
9	DNA/BIO	47,XX,+20[13]/46,XX[16]	-	-	-	-	-	-	-	CPM I	TOP[b]	46,XX

a = One of twin pregnancy, other twin displayed a normal karyotype.
b = TOP because of abnormal DNA results
For abbreviation see also Table 4, 5 and 6.

In cases 4 and 5, a twin pregnancy, both fetuses showed a mosaic 45,X/46,XX in STC-villi. Two colour FISH with X and Y probes on LTC-villi revealed one normal sample and one with an increased percentage nuclei with of one X signal. Subsequent amniocentesis was carried out for the differentiation of CPM from generalized mosaicism. Surprisingly, FISH on uncultured amniocytes revealed one normal XX and one normal XY fetus (Van den Berg et al., 1999).

In case No. 6, also a twin pregnancy, both STC- and LTC-villi karyotypes of one twin showed a XX/XY chimerism, while the other twin showed a normal 46,XY karyotype. After consulting the obstetrician, the chimerism was presumed to be caused by a sampling problem. Additional DNA analyis was performed and confirmed the hypothesis of the cross contamination. A healthy boy and girl were subsequently born.

In the other three cases (Nos 7, 8, and 9), where the uncertain results were due to an insufficient quality of the chromosomes, FISH on the metaphase level was carried out for identification and/or determination of derivatives with various probes. TOP was carried out in two cases (Nos 7 and 9) because of ultrasound abnormalities.

In one case (No. 10) FISH was performed for the identification of the *de novo* marker chromosome which turned out to be positive with a 13/21 centromeric probe and the whole chromosome paint (wcp) 13. Follow-up ultrasound investigation showed no abnormality and the pregnancy continued.

In case No. 11, FISH on STC-villi was applied with a 15psatIII specific probe to characterize the extra 15p material, which showed two signals in normal cells and only one signal in those cells with the der(15). Subsequent amniocentesis was carried out and FISH on uncultured amniocytes showed a normal signal distribution. Additional UPD investigations revealed a normal biparental chromosome 15 contribution.

Table 7 summarizes 14 cases with abnormal LTC-villi karyotypes, found after certain (normal or carrier) STC-villi (13 cases of CPM type II and one of CPM type III). Two cases revealed a non-mosaic chromosome aberration (Nos 2 and 5). It concerned an isochromosome 21 and a trisomy 8, respectively. In both cases FISH on LTC-villi slides turned out to be (non-mosaic) abnormal as well. To differentiate a GMDD from CPM type II, subsequent amniocentesis in both cases, in addition to a cordocentesis in case 5, was carried out and revealed normal results. The prenatal diagnosis of CPM type II could be made. Both pregnancies were continued.

All other cases revealed mosaicisms. FISH on interphase level was carried out in all cases, except in those cases involving a marker chromosome (Nos 3 and 4). Subsequent amniocentesis was carried out when FISH on LTC-villi slides was abnormal. All these cases turned out to be CPM type II and the pregnancies continued. In case 14, additional FISH on STC- and LTC-villi slides with a 16-probe showed an increased percentage (21%) of three signals whereas the STC-villi karyotype was normal. Amniocentesis was subsequently carried out and FISH (with the same 16-probe) on uncultured AF cells showed a normal signal distribution. The diagnosis of CPM III could be made and the pregnancy continued.

In case 3 and 4, displaying two cells with a marker chromosome CPM type II was assumed without follow-up investigation; a small risk of missing a GMDD-diagnosis was accepted. In case 9, the parents refrained from amniocentesis because of the small risk.

Table 8 illustrates those cases where LTC-villi were non-available and uncertain STC-villi karyotypes were found. In case 3, 4 and 8, FISH on STC-villi slides was carried out for the establishment of a more accurate level of mosaicism. This subsequent FISH investigation also resulted in the identification of the marker chromosome in case 4 which contained the X-centromere region. Subsequent amniocentesis was only carried out in cases 4 and 8 and not in case 3 because this involved a twin pregnancy and the small risk of missing a generalized mosaicism was taken. In case 7, parental blood investigation revealed a maternal t(11;22) but, unfortunately, the STC-villi karyotype remained uncertain and subsequent amniocentesis had to be carried out and showed a balanced 46,XY,t(11;22). The pregnancy was continued and a healthy male infant was born. In case 9, TOP was carried out because of abnormal DNA results. The trisomic 20 cell line was not confirmed in fetal fibroblasts.

In addition to the abnormalities listed in the Tables 4-8, 114 cases of tetraploidy mosaicism were encountered (Table 9). In 14 of these cases, follow-up ultrasound investigation was performed and in four amniocentesis, all with normal results. All these 14 cases were found in 1997 before the tetraploidy protocol was implemented (Figure 1) Thereafter, in only one case follow-up investigation took place in fetal fibroblasts after TOP because of unfavourable results of prenatal DNA analysis, the tetraploidy was not encountered in the fibroblasts.

During the period of this study, it was seen that if there were no analyses performed in LTC-villi preparations there would have been 53 cases with uncertain cytogenetic results (53/144 abnormal STC-villi; 36.8%). Conversely, the simultaneous analysis of STC- and LTC-villi introduced 14 (initially normal) cases with uncertain cytogenetic results. Nevertheless, the total number of uncertain results showed a significant reduction from 53/144 (36.8%) to 34/158 (21.5%) (χ^2=8.60, df=1; p<0.001). In comparison to our previous study on the analysis of STC-villi preparations alone (Los et al., 1998) with 110/219 (50.2%) uncertain results, the figure of 34/158 (21.5%) uncertain results of the present study represents a significant improvement (χ^2=32.02, df=1; p<0.001). This has not yet resulted in a significant decrease in the number of follow-up amniocentes over the period 1997-1999 (28/1829 CV samples) compared to that over the period 1993-1996 (74/3499). However, the combination of the implementation of the protocol for handling tetraploid cells and the high rate of obtaining a cytogenetic result after analysing both STC-and LTC-villi, resulted in a significant reduction of the number of follow-up amniocenteses from 74/3499 (2.1%) during the period 1993-1996 to 15/1225 (1.2%) in 1998-1999 (χ^2=3.89, df=1;p<0.05). After follow-up investigations, the 34 uncertain cases turned out to be CPMs in most instances (30/34) and a generalized abnormality in only four instances.

The distribution of the final cytogenetic results among the various indications is presented in Table 9. Frequencies of abnormal results are strongly correlated with the

indication for prenatal cytogenetic investigation in contrast to those of CPM cases which turned out to be independent from the a priori cytogenetic risk.
The frequencies of CPM type I, II, III, and CPM type I or III cases were 1.09% (20/1829), 0.71% (13/1829), 0.16% (3/1829) and 0.27% (5/1829), respectively.

The proportion of women with a low cytogenetic risk in the present study is significantly lower than that in the previous study (Los et al., 1998) (Table 10).

Table 9 - Distribution of the final cytogenetic results among the various indications

		Normal		Abnormal		CPM[b]				
Indication	N	0.583	4n/2n	Abn[a]	Carrier	I	II	III	I or III	Other
MA 36-39	954	853	63	11	6	11	5	1	3	1
MA ≥40	297	259	16	11	1	3	4	1	1	1
US Abn	157	90	6	56	2	1	1	1	-	-
Carrier	54	22	4	3	22	1	2	-	-	-
DNA/BIO	170	160	7	-	-	1	1	-	1	-
Other	197	173	18	1	2	3	-	-	-	-
All	1829	1557	114	82	33	20	13	3	5	2

a = Frequencies of abnormal results (US Abn and Carrier combined) differ significantly among the various indications ($\chi^2 = 317.01$, df = 4; $p < 0.001$).
b = Frequencies of CPM cases (US Abn and Carrier, DNA/BIO and Other combined) are evenly distributed ($\chi^2 = 1.07$, df = 3; $p > 0.05$)
2n = Only diploid cells; 4n/2n = Tetraploid and diploid cells.

Table 10 - Distribution of CV patients in high- and low cytogenetic risk indications

		Previous study 1993 - 1996	Present study 1997 - 1999
	Indications	N	N
High risk ≥3.5%	MA ≥40 US Abn Carrier	692	507
Low risk < 3.5%	MA 36-39 BIO/DNA Other	2807	1322

$\chi^2 = 43.07$,df=1 ; $p < 0.001$

Discussion

In a few large studies the relative accuracy of STC- and LTC-villi are compared (Ledbetter et al.,1990, 1992; ACC, 1994; Pittalis et al., 1994). The present study contributes more information about the accuracy of the analysis of both STC- and LTC-villi preparations.

The overall success rate for obtaining a cytogenetic diagnosis was 99.5% which is a good figure in view of the reported percentages (Ledbetter et al.,1992; Teshima et al., 1992; ACC, 1994).

The improvement of karyotype quality is evidently shown in Figure 2. Although analysing STC-villi alone did not lead to any overlooked chromosomal aberrations, analysing both STC- and LTC-villi clearly optimized the chromosome quality resulting in a quality about equal to that of AF cells.

The very low percentage of MCC in the present study (0.4%) is not only the result of careful dissection of the material, the culture method and the choice of appropriate medium (Saura et al., 1997; Smidt-Jensen et al., 1989), but is also due to the fact that all LTC-villi are harvested *in situ*, in our laboratory. There were no diagnostic errors due to MCC.

Since the introduction of the routine analysis of eight cells in LTC-villi additional to STC-villi, the number of routinely investigated cells in STC-villi cells has been reduced from 16 to 8. As a consequence of this, fewer cases of CPM type I and III were expected to be noticed. The observed prevalence of CPM type I and III was 1.5% of all CV samples, which is not significantly different from that of 2.0% obtained in our previous study (Los et al., 1998). The total number of cases with CPM type I, II and III (2.2%) matches the results of other studies (Leschot et al., 1996; Pittalis et al., 1994; Wang et al 1994).

The ultimate number of finally abnormal cytogenetic results in STC- and LTC-villi is somewhat higher than in previous studies (Ledbetter et al.,1992; Pittalis et al., 1994; Los et al., 1998). This is due to the fact that we advise chorionic villus sampling to those women with a high genetic risk (≥3.5%)(Los et al., 1998). It has been shown that this policy, together with the simultaneous analysis of STC- and LTC-villi preparations has led to a significant decrease in the proportion of uncertain results in comparison to our previous study. After the implementation of all guidelines for the handling of uncertain results, the follow-up amniocentesis rate declined significantly by one third, from 2.1% (1993-1996) to 1.2% (1998-1999). No incorrect diagnoses occurred and no false-negative results came to our attention. In four cases a small risk of uncertainty was accepted leading to a diagnostic accuracy of just below 100%.

The observation of a trisomy 8 mosaicism in CV is uncommon (Wang et al., 1994; Wolstenholme, 1996; Hahnemann and Vejerslev, 1997b). It is known that follow-up amniocentesis is not the method of choice to reveal trisomy 8 mosaicism (Klein et al., 1994; Schneider et al., 1994). Caution should therefore be exercised in the use of amniocentesis only. To minimize the risk of a false-negative result, follow-up

investigation for the differentiation between CPM type II and GMDD (case 5, Table 7) was carried out in simultaneously sampled amniotic fluid and fetal blood.

In patients with an X-autosome translocation, analysis of the X-inactivation pattern may help to delineate the clinical phenotype. It is known that the X-inactivation in these cases does not occur at random, but usually follows the pattern most favourable to the subject. In the case of a balanced t(X;aut), the normal X is usually inactivated, in contrast, in unbalanced t(X;aut) the translocated X is usually inactivated (Mattei et al., 1982; Bettio et al., 1994). About 95% of balanced and 91% of unbalanced t(X;aut) follow this most favourable model of inactivation. It is also known that the inactivation pattern of balanced X-autosome translocations may differ in lymphocytes and fibroblasts (Zori et al., 1993). We therefore studied X-inactivation patterns in LTC-villi, amniotic fluid cells, and fetal lymphocytes.

The importance of analysing both STC- and LTC-villi in cases with monosomy X is well known (Pittalis et al., 1994; Smith et al., 1999) especially for those cases without any ultrasound abnormalities. Only when both STC- and LTC-villi preparations show a 45,X karyotype, any degree of reliability can be assumed, where a monosomy X in STC-villi only, can be associated with normal and abnormal outcomes. However, the likelyhood of an abnormal outcome (generalised mosaicism) in cases of a low level 45,X/46,XX mosaicism in STC-villi, is quite low (Ledbetter et al., 1992). So, if LTC-villi preparations reveal a normal karyotype, follow-up investigations like amniocentesis are not indicated. However, follow-up amniocentesis or a postnatal confirmation in cord blood cells should be considered in those cases where LTC-villi are non-available and FISH on STC-villi shows abnormal signal distributions. In our study two cases of 45,X mosaicism, both twin pregnancies without LTC-villi (cases 2 and 3, Table 8), were seen. In case 2, amniocentesis was carried out to exclude a fetal mosaicism because a prenatally detected 45,X/46,XY mosaicism needs special attention. It is known that there is a risk of fetal abnormalities in such cases (Hsu et al., 1989; Chang et al., 1990; Van den Berg et al., 2000). In case 3, it was decided to perform a postnatal confirmation rather than taking any risks by instigatinga second invasive procedure because the chance of confirmation is low.

Experiences with 47,XXX, 47,XXY and 47,XYY are limited (Smith et al., 1999). We encountered one non-mosaic case of 47,XXX in STC-villi with a 46,XX karyotype in LTC-villi which was also confirmed in AF cells. Contrary to our previous study (Los et al., 1998) it is obvious that sex-chromosomal (non-mosaic) aneuploidies should be confirmed by amniocentesis if LTC-villi are not available.

In conclusion, prenatal cytogenetic diagnosis in STC-and LTC-villi demonstrates a very high laboratory success rate together with a quality improvement of the karyotype compared to that of STC-villi alone. No incorrect predictions were given and no misdiagnoses were made. It also demonstrates that there is an actual and significant reduction for the need of follow-up amniocenteses in comparison with the diagnosis in STC-villi alone. Our data confirm the high reliability of cytogenetic

results in both villi compartments. For pregnant women with a high cytogenetic risk, first-trimester CV sampling is a very good alternative for second trimester amniocentesis and might even be the prenatal test of first choice.

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Chapter 4

Prenatal diagnosis in multiple gestations

AMNIOCENTESIS OR CHORIONIC VILLUS SAMPLING IN MULTIPLE GESTATIONS ? EXPERIENCE WITH 500 CASES.

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Summary

500 women with multiple pregnancies underwent amniocentesis or chorionic villus (CV) sampling at our department between January 1988 and July 1997. The aim of this retrospective study was to evaluate the laboratory aspects and the consequences of discordant results in these pregnancies in relation to the method of sampling. Uncertain results in one or both samples, requiring further investigation were more frequent in CV samples (eight times in 163 paired samples, 5%) than in amniotic fluid (AF) samples (once in 298 paired samples, 0.3%). Sampling one fetus twice (erroneous sampling) was seen only once among 163 pregnancies with two CV samples in our study. Cross contamination due to mixed sampling was discovered in two of seven pregnancies that underwent DNA diagnosis in CV and might be a rather regular occuring phenomenen. In none of the 500 pregnancies mixed sampling caused diagnostic dilemmas. A third sampling problem, maternal cell contamination caused a diagnostic problem once among the AF samples. Selective fetal reduction appeared safer after CV sampling than after amniocentesis. Subsequently, CV sampling instead of amniocentesis became the method of choice for prenatal diagnosis in multiple pregnancies in our department.

Introduction

Multiple gestations present particular counselling, technical and management problems in prenatal diagnosis, especially in case of discordancies (Brambati et al.,1991; Christiaens et al.,1994; Brandenburg et al.,1994). Hunter and Cox (1979) were the first to point out the raised genetic risk in twin pregnancies compared with singleton pregnancies. This is of particular importance because dizygotic twinning increases as a function of advancing maternal age and by the extended use of assisted procreation techniques. Genetic prenatal diagnosis is mostly performed because of advanced maternal age.

The genetic and/or laboratory aspects of amniotic fluid (AF) and chorionic villus (CV) sampling in multiple pregnancies have not been explored in depth in any large series. Most articles concern the increased risk of fetal loss for women undergoing amniocentesis in multiple gestations (Anderson et al.,1991; Pruggmayer et al., 1991, 1992). To our knowledge there are only a few studies which compare the genetic aspects of second-trimester amniocentesis and first-trimester CV sampling for multiple gestations (Wapner et al.,1993; Brandenburg et al.,1994).

Since 1992 we prefer CV sampling over amniocentesis in twin pregnancies because of the lower risk of first trimester selective fetal reduction (SEL RED) in case of discordant results. The encounter of a case of confined placental mosaicism (CPM) in both fetuses of a twin pregnancy due to erroneous sampling, one placenta was inadvertently sampled twice, made us decide to review our experience with genetic amniocentesis and CV sampling in multiple pregnancies. Our main objective was to investigate the accuracy and reliability of both procedures and what recommendations could be made in this group of patients.

Materials and Methods

Patients

Between January 1988 and July 1997, 482 twin and 18 triplet pregnancies were observed in a total of 25.850 genetic AF and CV samples in our department. The records of all women with multiple pregnancies who underwent genetic amniocentesis and/or CV sampling over that period were studied and reviewed.

The indications for prenatal diagnosis in these multiple pregnancies were advanced maternal age (≥36 years) (MA), risk for neural tube defects (NTD), parental carriership for chromosomal abnormality (Carrier), ultrasound abnormalities (US), recurrence risk for various metabolic diseases and Mendelian inherited diseases detectable with DNA analysis (BIO/DNA), previous child with (chromosomal) abnormality (recurrence risk, RR), other reasons (Other) and follow-up investigations in amniotic fluid because of uncertain abnormalities in a previous CV sample (Follow-up).

Obstetrical methods

At the initial visit each patient had an obstetric and genetic assessment. The obstetric assessment included a real-time ultrasound examination to monitor the number of fetuses, fetal heart rate, crown-rump length or fetal biparietal diameter and to establish the localisation and the number of the placentae. Furthermore, the thickness of the septum between the amniotic sacs and the presence of the lambda sign or T-sign were evaluated (Kurtz et al.,1992; Wapner et al.,1993). All patients were given information about the available diagnostic procedures, the present knowledge of the risks involved and the difficulties related to the selectivity in sampling. The patients were also informed about the risks of anomalies in multiple gestations, the possibility that the fetuses may be discordant for an abnormality (Hunter and Cox et al., 1979) and the risks of selective feticide (Brandenburg et al.,1994).

All amniocenteses were performed by experienced obstetricians under direct ultrasound guidance as described previously (Pijpers et al.,1988). Since August 1990 the identification of the amniotic sacs has been made by ultrasound only. The use of intra-amniotic dye was abandoned since a relationship between the use of methylene blue and the occurrence of jejunal atresia was suspected (Nicolini and Monni,1990; Van der Pol et al.,1992; Brandenburg et al., 1997). Approximately 15-20 ml of amniotic fluid was aspirated from each amniotic cavity. In cases of biochemical or DNA investigations 30 ml was aspirated. When amniocentesis was not possible because of oligohydramnios or anhydramnios, fetal blood (FB) sampling was performed as previously described (Den Hollander et al.,1994).

All transabdominal CV sampling procedures were carried out under continuous ultrasound guidance by experienced obstetricians (Jahoda et al.,1991). In pregnancies where two distinct placental sites were separated by a thick membrane, each placental site was sampled individually. In pregnancies with only one distinct placental mass and an identifiable thin, wispy membrane an area near each cord insertion was sampled. If there was only one placental mass and no membrane could be seen and the cord sites were not distinguishable, only one sample was taken.
SEL RED after discordant results in CV and amniotic fluid was carried out with intracardiac infusion of KCl 15% (Golbus et al., 1988; Berkowitz et al.,1993).

Laboratory methods

The chromosomal analysis of AF cells was performed by the *in situ* method on glass coverslips. CV were incubated overnight using fluorodeoxyuridine (FdU) synchronization (short term culture; STC)(Gibas et al.,1987). Since 1997 long-term cultures (LTC) have been initiated simultaneously when at least 20 mg was sampled (Smidt-Jensen et al.,1989). Metaphase spreads of fetal lymphocytes were performed according to standard techniques.
Trypsin-Giemsa staining was routinely used for karyotyping AF cells, CV cells and fetal lymphocytes and a minimum of 16 cells/clones were analysed in each case.

Since 1996, interphase fluorescence *in situ* hybridization (FISH) has routinely been performed according to standard protocols (Van Opstal et al.,1993; 1998b) with a probe-set for the chromosomes 13, 18, 21, X, Y on uncultured amniocytes of all multiple pregnancies in case CV sampling was not possible for genetic or technical reasons, or because of a gestational age beyond the appropriate time for CV sampling. FISH was also performed on STC-villi, LTC-villi and AF slides when follow-up investigations were needed for the differentiation between pseudomosaicism and true mosaicism or between CPM and generalized mosaicism. (Van den Berg et al.,1997; Van Opstal 1998a; Los et al.,1998).

Alpha-fetoprotein (AFP) measurements were done in our department with radial immunodiffusion (Kleijer et al.,1978). In cases of equivocal or non-understood raised AFP levels, acetyl-cholinesterase (AChE) banding was performed additionally (Brock, 1992), and ultrasound investigation carried out.

Prenatal biochemical and DNA analysis were done according to techniques as described previously (Galjaard and Kleijer,1986; Wanders et al.,1996) and according to standard methods.

Statistics

The Fisher exact test was used for the statistical analysis of differences between the numbers of certainly and uncertainly abnormal cytogenetic results in AF and CV, and for the evaluation of the success rate of SEL RED after amniocentesis and CV sampling.

Results

In the group of 500 multiple gestations there were 482 sets of twins and 18 sets of triplets. 163 women with a twin pregnancy were successfully sampled twice by CV sampling (Table 1) and 298 women underwent amniocentesis for both fetuses (Table 2). In 28 twin pregnancies two different samples were received or only one sample (Table 3). The reason for just one sample was mono-chorionicity in seven cases, mono-amnionicity in eight cases and unaccountable in six cases. The reason for two different samples (one AF and one CV or FB sample) was because of anhydramnion in one of the two compartments in all seven cases. In eight pregnancies repeat sampling was performed because of uncertain laboratory results.

Four women with a triplet pregnancy were successfully sampled by CV sampling, 11 underwent amniocentesis. One of these latter triplets showed two monozygous fetuses in one amniotic sac and a third dizygous fetus in a second amniotic sac, so two compartments were sampled. There was one triplet pregnancy where one CV and three AF were sampled. Owing to oligohydramnios in one of the compartments it was only possible to aspirate enough AF for AFP measurements, which necessitated CV sampling for chromosome analysis (Table 4).

Table 1 - Indications for chorionic villus sampling in twin pregnancies with two samples

Indication	Number of pregnancies	Pregnancies with normal results in both samples	Pregnancies with abnormal results in one or two samples	
			Number	%
MA	116	107	9	8
US	9	5	4	44
RR	12	12	0	0
Other	10	9	1	10
BIO/DNA	13	5	8	62
Carrier	3	0	3	100
Total	163	138	25	15

Table 2 - Indications for amniocentesis in twin pregnancies with two samples

Indication	Number of pregnancies	Pregnancies with normal results in both samples	Pregnancies with abnormal results in one or two samples	
			Number	%
MA	168	160	8	5
NTD	47	44	3	6
US	40	21	19	48
RR	21	21	-	-
Other	6	6	-	-
BIO/DNA	8	6	2	25
Carrier	2	0	2	100
Follow-up	6 (1)[a]	4 (1)[a]	2	40
Total	298	262	36	12

a = follow-up after uncertain result in earlier AF sample

Table 3 - Indications for amniocentesis and chorionic villus sampling in twin pregnancies with only one sample or two different samples

Indication	Number of pregnancies with one sample		Number of pregnancies with different samples		Normal pregnancies	Abnormal pregnacies	
	AF	CV	AF+CV	AF+FB		Number	%
US	10[a]	2[b]	3	4	14	5	26
MA	2[c]	3[b]	-	-	5	-	-
NTD	1[b]	-	-	-	1	-	-
RR	-	1[b]	-	-	1	-	-
Other	-	1[b]	-	-	1	-	-
Follow-up	1				-	1	100
Total	14	7	3	4	22	6	21

AF = amniotic fluid; CV= chorionic villus; FB = fetal blood; a = six are mono-amniotic; b = all mono-amniotic/ monochorionic, c = one is mono-amniotic

Table 4 - Indications for amniocentesis and/or chorionic villus sampling in triplet pregnancies

Indication	Number of pregnancies with samples consisting of				Number of pregnancies with	
	3AF	3CV	3AF 1CV	2AF	Normal results in all investigated samples	Abnormal results in ≥ 1 samples
MA	11	4	-	-	15	0
US	1	-	1	1	1	2
Total	12	4	1	1	16	2

Cytogenetic, biochemical and DNA analyses, CV group with two samples. (Table 1, 5 and 6)

In the 163 twin pregnancies undergoing CV sampling, cytogenetic investigations were performed in 161 twin pregnancies; in one pregnancy, only one karyotype could be achieved. The two pregnancies without cytogenetic investigations concerned cases where biochemical analysis was indicated and the amount of villi was not enough to perform cytogenetic analysis as well. The success rate of obtaining a cytogenetic, biochemical and DNA result of each fetus sampled was 99.7, 100 and 100%, respectively.

Table 5 - Abnormal karyotypes in the group with 2 chorionic villus samples

Case	Indication	Twin A	Twin B	Outcome twin A	Outcome twin B
1	MA	**47,XYY**	46,XY	Normal ♂	Normal ♂
2[a]		**47,XY,+21**	46,XX	SEL TOP	Normal ♀
3[b]		**45,X**	46,XY	AF direct FISH: X/XY, SEL RED	Normal ♂
4		**92,XXXX[9]/46,XX[22]**	46,XX	Normal ultrasound, normal ♀	Normal ultrasound, normal ♀
5		**45,X[6]/46,XY[24]**	46,XY	AF direct FISH: XY, normal ♂	AF direct FISH: XY, normal ♂
6		**47,XX,+mar[23]/46,XX[16]**	46,XX	AF: 47,XX,+mar[12]/ 46,XX[7], normal ♀	AF: 46,XX , normal ♀
7		**48,XY,+7,+13[40]/ 47,XY,+7[2]/46,XY[8]**	46,XY	AF: 46,XY, normal ♂	AF: 46,XY, normal ♂
8		**47,XX,+21**	46,XX	SEL RED	Normal ♀
9		**45,X[20]/46,XX[7]**	**45,X[28]/46,XX[2]**	AF: 46,XX, normal ♀	AF: 46,XY , normal ♂
10	Other	**46,XX,t(6;13)[6]/46,XX[34]**	46,XY	AF: 46,XX , 34 weeks, normal ♀	AF: 46,XY, 34 weeks, schizis ♂
11	US	**47,XX,+21**	46,XX	SEL RED	33 weeks, normal ♂
12		**45,X[2]/46,XX[18]**	46,XX	AF: 46,XX, 28 weeks, normal ♀	AF: 46,XX, AFP↑, IUD,
13		**47,XX,+18**	46,XY	SEL RED	omphalocele
14		**47,XY,+21**	46,XX	SEL RED	Normal ♂ Normal ♀
15	Carier	**45,XY,t(14;21)(q10;q10)mat**	46,XX	36 weeks, normal ♂	33 weeks, normal ♀
16		**45,XY,t(14;21)(q10;q10)mat**	46,XY	33 weeks, normal ♂	33 weeks, normal ♂
17		**46,XX,t(14;19)(q24.1;q13.4)pat**	46,XY	Normal ♀	Normal ♂

AF = amniotic fluid, FISH= fluorescence *in situ* hybridization, a = previously described by in't Veld et al. (1995); b = previously described by Los et al. (1998)

Cytogenetic abnormalities

Certainly discordant, uncertainly discordant and uncertainly concordant abnormal results were detected in 17 twin pregnancies (10.5%) (Table 5).
Certainly discordant results were detected in nine pregnancies (numbers 1, 2, 8, 11, 13, 14, 15, 16, 17); in six of these one fetus exhibited aneuploidy while the other was normal. Based on this information five couples requested SEL RED and one couple chose to continue the pregnancy because of the minimal phenotypic expression of the chromosomal abnormality (number 1). SEL RED in these five pregnancies was successful and resulted in the delivery of normal infants. The other three certainly discordant results concerned parental translocation carriers.

Uncertainly discordant results were achieved in seven pregnancies (numbers 3, 4, 5, 6, 7, 10, 12). In one case (number 3), one fetus exhibited a 45,X karyotype. The possibility of a CPM was discussed and an early amniocentesis at 12.5 weeks of gestation with rapid investigation on uncultered AF cells was recommended. Two-colour FISH with X and Y probes showed a X/XY mosaicism and after counselling the parents requested SEL RED, which was successful and resulted in the delivery of a normal boy. Chromosomal mosaicism was found in the other six pregnancies (numbers 4, 5, 6, 7, 10, 12). Additional investigations such as ultrasound and follow-up amniocentesis were performed and the mosaicism indicated CPM in five of the six cases; in case 6, mosaicism of a *de novo* marker chromosome was confirmed in AF cells. After genetic counselling and normal ultrasound findings the parents chose to continue the pregnancy.

An uncertainly concordant abnormal result was achieved in one pregnancy (number 9). In this case both fetuses showed a mosaic 45,X/46,XX in STC-villi preparations. Two-colour FISH with X and Y probes on LTC-villi showed an increased percentage of interphase nuclei with only one X signal in fetus B. The other fetus (A) turned out to be normal XX.
The possibility of a real mosaicism in one fetus was discussed and amniocentesis was recommended. FISH on unculteredamniocytes revealed normal XX results in fetus A and normal XY results in fetus B; 46,XX and 46,XY karytypes were established in cultured AF cells. The parents were informed and after an extra ultrasound examination showing one normal female and one normal male fetus, the pregnancy continued. A normal girl and normal boy were born after 37 weeks of gestation.

Biochemical and DNA analysis

These were performed in six and seven twin pregnancies, respectively. Concordant normal results were encountered in five cases (38%); discordant results in six (46%) and concordant abnormal results in two cases (15%)(Table 6).
SEL RED was requested and carried out in five of the six cases of discordancy, resulting in the birth of five normal infants. In the case of suspected discordant pyruvate carboxylase (PC) activities termination of pregnancy (TOP) was decided on elsewhere. Fetal fibroblasts cultures were received from both fetuses; twin B had

normal PC activity as predicted, whereas twin A had reduced but not absent activity, suggesting heterozygosity rather than an affected fetus.
In two of the seven prenatal DNA investigations we encountered admixture of CV samples, as one DNA sample represented one fetus and the other sample contained DNA of both fetuses (cross contamination)(Fig.1).

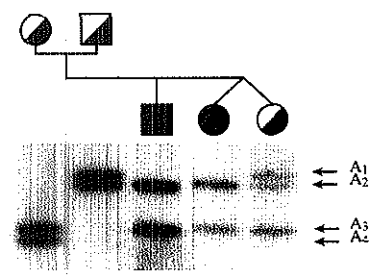

Figure 1 - Example of cross contamination (admixture of villi of one fetus to that of the other) in a twin pregnancy with discordant results of cystic fibrosis (CF) mutation (ΔF508) analysis. The admixture was demonstrated with a DNA-marker independent from the CF; YNZ22.1 (chromosoome 17). The homozygous fwtus displays the maternal allele A_3 and the paternal allele A_2, the heterozygous fetus displays the maternal allele A_3, the genuine paternal allele A_2 and the admixed paternal allele A_2.

Table 6 - Abnormal BIO and DNA results found in twin pregnancies with two CVS or AF samples

Indication	Twin A		Twin B		Outcome Twin A	Outcome Twin B
DNA:						
Cystic Fibrosis	46,XY	A	46,XY	A	TOP	TOP
	46,XX	A	46,XX	N	SEL RED	Normal ♀
	46,XX	A	46,XY	N	SEL RED	Normal ♂
Fragile X syndrome	46,XX	A	46,XX	N	SEL RED	Normal ♀
X-linked Retinoschizis	46,XY	A	46,XX	N	SEL RED	Normal ♀
BIO:						
Arginino succinuria [a]	46,XX	A	46,XY	A	TOP	TOP
Hurler syndrome	46,XY	A	46,XY	N	SEL RED	Normal ♂
Pyruvate carboxylase (PC) deficiency [b]	46,XY	A	46,XX	N	TOP, low PCactivity, probably heterozygote	TOP, normal PC activity
Cystic Fibrosis [c]	--,--	A	--,--	A	no follow-up	no follow-up

a = previously described by Pijpers *et al.*(1990); b = karyotyping in CV samples elsewhere performed; c = AF, others are CV samples.

Cytogenetic, AFP, biochemical and DNA analyses, AF group with two samples (Table 2, 6, and 7)

In the 298 twin pregnancies undergoing amniocentesis, cytogenetic investigations were performed in 294 twin pregnancies and successful in 292. In two pregnancies no karyotype was obtained in one of both samples due to growth failure. So, the success rate of obtaining a cytogenetic result of each fetus was 99.3%. The success rate of obtaining AFP, biochemical and DNA results of each fetus sampled was 100%.

Cytogenetic abnormalities

Discordant and concordant abnormal results were detected in 15 pregnancies (5%) (Table 7). Discordant abnormal results were detected in 12 pregnancies. In four of these pregnancies one fetus exhibited aneuploidy while the other was normal (numbers 3, 4, 5, 6). Based on this information three couples opted for SEL RED (numbers 3, 5, 6) and one couple chose to continue the pregnancy because of the mild phenotypic expression of the chromosome abnormality (number 4). In three pregnancies there was a discordancy for triploidy while the other was normal (numbers 19, 28, 31). The pregnancies were too advanced for SEL RED and pregnancy outcome is shown in Table 7. In five pregnancies (numbers 7, 13, 15, 34, 35) discordant structural abnormalities were found. In one pregnancy (number 7) one fetal karyotype showed an inversion. Chromosome analysis of blood lymphocytes of both parents demonstrated the inversion to be familiar. In case 15, it concerned a mosaicism of a *de novo* marker chromosome, encountered in a previous CV sample (case 6 of Table 5). In the cases 13 and 34 both fetuses showed abnormal karyotypes, one a balanced and the other one an unbalanced parental translocation. In case 13 the parents opted for SEL RED; unfortunately the pregnancy ended two weeks after the SEL RED procedure, at 21 weeks of gestation. In the other case the pregnancy was too advanced for SEL RED. This pregnancy ended with IUD of the affected fetus with the unbalanced karyotype and the delivery of a normal girl with the balanced karyotype at 37 weeks of gestation. In the last discordant case (number 35) there was a discordancy for Cri du Chat syndrome. After counselling the parents (both with normal karyotypes) opted for SEL RED at 19 weeks' gestation. Unfortunately, the pregnancy ended immature at 21 weeks' gestation, after the reduction procedure.

Concordant abnormal cytogenetic results were detected in three pregnancies (numbers 8, 12, and 14). In case 8 both fetuses showed a 46,XX/46,XY mosaicism, representing an uncertainly abnormal concordant result. The possibility of maternal cell contamination or a cross-contamination was discussed and further diagnostic procedures were recommended such as ultrasound examination and repeat amniocentesis. Ultrasound examination at the time of the repeat amniocentesis revealed two male fetuses without abnormalities. Chromosome analysis yielded normal male karyotypes and the pregnancy continued; two normal boys were born.

In the other two cases the concordant abnormal results demonstrated parental translocation carriers.

AFP abnormalities

Abnormal AFP levels in one or both AF samples were detected in 24 pregancies (8%) (Table 7). Certainly discordant AFP results were detected in six pregnancies (numbers 2, 16, 20, 27, 32 and 34); five with both normal karyotypes and one with both abnormal karyotypes. US abnormalities were present in all fetuses from the AF compartment with high AFP levels. In case 27, both fetuses showed US abnormalities.

Uncertainly discordant AFP levels were present in three cases (numbers 1, 10 and 11). In one case (number 1) the patient had undergone *in vitro* fertilization (IVF) and the initial number of four fetuses was reduced to two. The high level of AFP in one compartment was assumed to be caused by AFP diffusion out of (one of) the reduced compartments. In another case (number 10) the AFP level was equivocal for 16 weeks of gestation. Additional US investigation showed a normal fetus and a gestational age of only 15 weeks and the AFP level turned out to be normal after correction. The high level of AFP in case 11 was explained by fetal blood contamination.

Concordant abnormal results were detected in 15 pregnancies. Abnormal AFP levels in both compartments with normal karyotypes were detected in 12 pregnancies (Table 7). Seven of these pregnancies (numbers 9, 22, 23, 24, 25, 26 and 29) showed ultrasound abnormalities of both fetuses which explained the elevated levels. In the other five cases (numbers 17, 18, 21, 30 and 33) only one fetus showed US abnormalities and the elevated AFP level of the AF compartment of the other normal fetus was assumed to be caused by AFP diffusion through the amniotic membranes. Besides the concordant abnormal AFP results there was a discordancy for triploidy in three pregnancies (numbers 19, 28 and 31).

Biochemical and DNA analysis

These were performed in eight and two twin pregnancies, respectively. Concordant normal biochemical results were encountered in seven cases (87%) and concordant abnormal biochemical results in one case (13%). This latter case escaped from our folluw-up. Prenatal DNA analyses were not performed in AF samples on *a priori* known DNA indications; in two cases, analysis was performed in addition to cytogenetics and AFP measurements for the establishment of zygosity.

Table 7: Abnormal results (cytogentics and/or AFP measurement) found in twin pregnancies with two amniotic samples

			Twin A		Twin B			
Case	Indication	GA	Karyotype	AFP	Karyotype	AFP	Outcome twin A	Outcome twin B
1	MA	16	46,XY	A	46,XY	N	35 weeks, normal ♂	35 weeks, normal ♂
2		16	46,XY	A	46,XY	N	IUD, anencephaly	35 weeks, normal ♂
3		16	47,XY,+21	N	46,XY	N	19 weeks, SEL TOP	34 weeks, normal ♂
4		16	47,XXX	N	46,XX	N	Normal ♀	Normal ♀
5[a]		16	47,XX,+21	N	46,XY	N	19 weeks, SEL TOP	38 weeks, normal ♂
6		17	47,XY,+21	N	46,XX	N	SEL TOP	37 weeks, normal ♀
7		16[5]	46,XY,inv(3)(p13p25)	N	46,XY	N	Normal ♂	Normal ♂
8		16	46,XX[12]/46,XY[7]	N	46,XX[3]/46,XY[9]	N	Second AF: 46,XY, 28 weeks, normal ♂	Second AF: 46,XY, 28 weeks, normal ♂
9[b]	NTD	18[5]	46,XY	A	46,XY	A	TOP, ♂ myelocèle	TOP, ♂ myelocèle
10		16	46,XY	A	46,XY	N	37 weeks, normal ♂	37 weeks, normal ♂
11		16	Elsewhere	A	Elsewhere	N	Normal ♀	Normal ♀
12[c]	DNA/BIO	14[5]	46,XY,inv(11)(q21q23)	N	46,XY,inv(11)(q21q23)	N	Normal ♂	Normal ♂
13	Carrier	17	46,XY,der(9)t(9;12)(p22;p11.2)mat	N	46,XX,t(9;12)(p22;p11.2)mat	N	19 weeks, SEL RED	21 weeks, immature delivery, ♀, no abnormalities
14		17	47,XY,+i(15p)mat	N	47,XY,+i(15p)mat	N	Normal ♂	Normal ♂
15	Follow-up	17	47,XX,+mar[12]/46,XX[7]	N	46,XX	N	35 weeks, normal ♀	35 weeks, normal ♀
16		14	46,XX	N	46,XX	A	28 weeks, normal ♀	IUD, omphalocele
17	US	23	46,XY	A	46,XY	A	IUD, MCA	Normal ♂
18		23	46,XX	A	46,XY	A	34 weeks, anencephaly	34 weeks, normal ♂
19		21[5]	69,XXX	A	46,XY	A	32 weeks, IUD	36 weeks, normal ♂
20		27[5]	46,XY	A	46,XY	N	32 weeks, omphalocele, pp†	32 weeks, normal ♂
21		26	46,XX	A	46,XX	A	26 weeks, IUD, mediastinal teratoma	26 weeks, normal ♀
22		22	46,XY	A	46,XY	A	24 weeks, TOP, anencephaly	24 weeks, TOP, congenital heart defect
23		24	46,XY	A	46,XY	A	33 weeks, pp†, body stalk anomaly	33 weeks, ♂, microcephaly, epileptic
24		20[5]	46,XX	A	46,XX	A	22 weeks, TOP, NTD	22 weeks, TOP,congenital heart defect
25		22[3]	46,XX	A	46,XX	A	31 weeks, IUD, MCA	31 weeks, IUD, twin to twin transfusion syndrome
26		21	46,XY	A	46,XY	A	25 weeks, pp†, hydrops	25 weeks, pp†, hydrothorax
27		19	46,XX	A	46,XX	N	19 weeks, IUD, twin to twin transfusion syndrome	19 weeks, IUD, acardiacus
28		24[4]	69,XXY	A	46,XX	A	32 weeks, pp†	32 weeks, normal ♀
29		31[3]	46,XY	A	46,XY	A	32 weeks ♂, duodenal atresia	32 weeks ♂, esophageal-atresia
30		26[4]	46,XX	A	46,XX	A	36 weeks, pp†, omphalocele	36 weeks, normal ♂
31		21[5]	69,XXX	A	46,XY	A	pp†	Normal ♂
32		28[3]	46,XY	A	FISH XX	N	33 weeks, ♂, teratoma	33 weeks, normal ♀
33		25[6]	46,XY	A	46,XY	A	37 weeks, ♂, dilatated ureter	37 weeks, normal ♂
34[d]		26	46,XY,+13,der(13;14)(q10;q10)	A	45,XX,der(13;14)(q10;q10)	N	IUD, MCA	37 weeks, normal ♀
35		16	46,XX,del(5)(p14→pter)	N	46,XY	N	19 weeks, SEL RED	21 weeks, immature delivery, ♂, no abnormalities

GA = gestational age in weeks; a = previously described by Pijpers et al. (1989); b = previously described by Omtzigt et al. (1992); c = normal biochemical results; d = previously described by Veld et al. (1997)

Cytogenetic and AFP analyses, group with only one or two different samples (Table 3)

In this group of twin pregnancies the success rate of obtaining a cytogenetic result of each fetus sampled was 100%. AFP measurements were successfully performed in all AF samples.

Cytogenetic abnormalities

These were detected in two pregnancies (7%). One concordant abnormal cytogenetic result was detected in the case where one AF and one FB sample was performed. Both fetuses showed a 47,XX,+18 karyotype. After genetic counselling the couple opted for termination of pregnancy. A discordant abnormal cytogenetic result, previously detected in CV, was confirmed (case 3, Table 5).

AFP abnormalities

Abnormal AFP levels were detected in four pregnancies (14%). One mono-amniotic pregnancy concerned a normal fetus and a fetus acardiacus which explained the abnormal AFP level. The pregnancy ended with IUD of the affected fetus and the birth of one normal child. The other three pregnancies concerned discordancy in AF volume (severe oligohydramnios in one and gross polyhydramnios in the other AF compartment). The abnormal AF volumes and AFP levels were assumed to be caused by twin to twin transfusion. In two pregnancies IUD occurred of both fetuses at 24 weeks'gestation. The third pregnancy ended with IUD of one fetus and the birth of one normal child.

Cytogenetic and AFP analyses, group of triplet pregnancies (Table 4)

In 18 triplet pregnancies in which AF and/or CV sampling was performed the success rate of obtaining a cytogenetic result of each fetus sampled was 100%. AFP measurements were successful performed in all AF samples.

Cytogenetic abnormalities

Only one discordant abnormal result was detected in this group of patients (5.6%). It concerned a pregnancy in which one CV and three AF samples were performed. The fetus with oligohydramnios exhibited a trisomy 21 while the other two fetuses were normal. After genetic counselling the couple decided to continue the pregnancy. At 33 weeks of gestation the pregnancy ended with the delivery of three infants; the one with trisomy 21 died 10 minutes post partum.

AFP abnormalities

One concordant abnormal result was detected in a triplet pregnancy which showed two monozygous fetuses (A and B) in one compartment and a third fetus (C) in another compartment. The abnormal AFP levels could be explained by the presence of two fetuses in one AF compartment, one fetus (B) with obstructive uropathy and hydronephrosis in fetus C. At 32 weeks of gestation the pregnancy ended with IUD of fetus B and the delivery of two normal boys. The hydronephrosis seen in fetus C was not recovered after birth, so it concerned a transient hydronephrosis.

The rate of certain : uncertain abnormal cytogenetic results was significantly higher in AF samples than in CV samples (p=0.018).
In 16 pregnancies a SEL RED was carried out; five at 18-19 weeks' gestation after discordant results in AF; three successful and two resulting in loss of both fetuses (40%). Selective reduction in the other 11 pregnancies was successfully carried out at 12-14 weeks' gestation and these pregnancies ended with the birth of single healthy children.
SEL RED seemed safer after early prenatal diagnosis in CV than after second-trimester prenatal diagnosis in AF cells, but this difference was not significant (p=0.08).

Discussion

Prenatal diagnosis in multiple pregnancies is more complex than in singleton pregnancies due to some specific circumstances and potential situations:
(1) increased risk of fetal loss (Anderson et al.,1991); (2) certainly and uncertainly discordant results; (3) certainly and uncertainly concordant results; (4) SEL RED in cases of certainly discordant results in CV or AF; (5) complex counselling.

Discordant results with the option of SEL RED of the affected fetus are to be expected in a high frequency in the prenatal diagnosis of Mendelian inherited diseases (DNA and biochemical investigations) (37.5% in autosomal recessive diseases and 50% in autosomal dominant diseases), and in a lower frequency in prenatal cytogenetics (Fig. 2). Furthermore, Fig. 2 shows the theoretical figures for uncertainly discordant or concordant results requiring further investigation. Uncertainly discordant results were observed in 7/160 twin pregnancies (4.4%) cytogenetically investigated in CV samples and expected in 2.8%; two cases turned out to be generalized mosaicism (1.3%) while CPM was found in five cases (3.1%). Certainly discordant results were encountered in 9/160 twin pregnancies (5.6%), which equals the expected figure of 5.9%.

In AF samples, no uncertainly discordant cytogenetic results were observed (expected frequency 0.4%). However, in the AF group as well as in the CV group, one pregnancy showed uncertainly concordant karyotypes which is theoretically rare (expected frequency in CV samples 0.02% and in AF samples 0.0004%).

A

Fetus B \ Fetus A	N	GM	CPM	ABN
N	91	0.19	1.2	3.0
GM	0.19	0.0004	0.0025	0.0062
CPM	1.2	0.0025	0.017	0.04
ABN	3.0	0.0062	0.04	0.096

Chorionic Villi

B

Fetus B \ Fetus A	Normal	Uncertain	Abnormal
Normal	95	0.195	2
Uncertain	0.195	0.0004	0.0046
Abnormal	2	0.0046	0.0529

Amniotic Fluid

Figure 2 - **(A)** Probabilities (in%) of concordant and discordant cytogenetic results in CV samples in both fetuses of a dizygote twin pregnancy; the frequencies of normal (N; 95.4%), abnormal (ABN; 3.1%), generalized mosaicism (GM; 0.2%) and confined placental mosaicism (CPM; 1.3%) are taken from Pittalis et al. (1994). **(B)** Chance (in%) of discordancies in AF samples of dizygote twins. Abnormality rate is 2.3% (Ferguson-Smith and Yates, 1984). Rate of uncertain results requiring further investigation is 0.2% (our own figures, 10 times in 5265 cytogenetic investigations)

In such a case one should be suspicious of a sampling problem as the cause of the unprobable results (Brambati et al., 1991). However, another cause might be monozygozity of the fetuses.
Significantly more cytogenetically uncertain results emerged in CV samples than in AF samples, eight (5%) versus one (0.3%). Finally, no diagnostic errors were made.

Concordantly raised AF-AFP levels were encountered in 15 twin pregnancies. In 8 of these 15 twins (53%) only one fetus was abnormal; the AFP level in the AF compartment of the normal fetus is assumed to be elevated by diffusion through the membranes out of the abnormal compartment (Franke and Estel, 1978; Stiller et al.,1988). Discordant AFP levels were seen in nine pregnancies (3%), with an abnormal fetus in the AF compartment with the abnormal AFP level in five cases. In one pregnancy with two abnormal fetuses, the normal AFP level represented a false negative finding. In three pregnancies with two normal fetuses, the raised levels could be explained by circumstantial factors other than fetal malformations. However, sometimes, raised AF-AFP levels are found in twin pregnancies without explanation (Brock et al., 1975).

Discordant and concordant results in the prenatal DNA and biochemical investigations were within the expected ranges. The numbers are to small for speculations about possible uncertain results. However in one case of prenatal PC activity measurement in CV, a diagnostic error occurred due to very low PC activity in the villi which hampered the discrimination between an affected and a heterozygous fetus.

Discordant or concordant results can be uncertain by two potential events; laboratory findings as CPM or pseudomosaicism and by sampling problems. Erroneous sampling, mixed sampling resulting in cross contamination and maternal cell contamination are three sampling problems which can be expected. It is known that in multiple pregnancies there are no guarantees that each fetus is sampled

individually. We did not encounter diagnostic problems in cytogenetics due to erroneous sampling or cross-contamination in AF samples.
However, one case with a concordant 46,XX/46,XY mosaicism, presumed to be caused by mixed sampling turned out to be caused by maternal cell contamination of both AF samples.

CV sampling in multiple pregnancies entails a greater risk of mixed samples than with AF sampling, approximately in 4-6% of the patients (Brambati et al.,1984, 1991; Pergament et al., 1992; Wapner et al., 1993). In our CV samples we had no diagnostic problems concerning cytogenetics due to mixed samples. However, in two of the seven pregnancies in which DNA analysis was carried out cross-contamination was established without causing diagnostic problems. This indicates that cross-contamination might be a frequently occurring and theoretically a diagnosis disturbing phenomenon. It is especially dangerous in prenatal biochemical analysis, where cross-contamination of villi of the affected fetus with those of the normal fetus might lead to false negative biochemical findings. So, in prenatal biochemical diagnosis in multiple pregnancies, additional DNA analysis is strongly recommended in order to detect potential cross contamination. In our series we have had noticeable erroneous sampling in only one of the 163 cases (0.6%), which is not significantly different from the reported figure of 2 out of 128 (1.6%) multiple pregnancies by Pergament et al. (1992).

Discordancy can be managed in three possible ways; (1) termination of the whole pregnancy; (2) SEL RED of the affected fetus; (3) a non interference policy of the affected fetus by continuation of pregnancy. We have seen all these three possibilities in our series of 500 multiple pregnancies. Although the difference between SEL RED after CV sampling and amniocentesis is not statistically significant, we believe that prenatal diagnosis at an early stage (11-12 weeks) enables a safer SEL RED in the case of discordant results than at 16 weeks. The risk of complications after SEL RED is less when it is carried out around 12 weeks' gestation rather than at 16 weeks or later (Golbus et al., 1988; Evans et al., 1991).

Comparing amniocentesis and CV sampling for the prenatal diagnosis in multiple pregnancies three major conclusions may be drawn; (1) there is a greater risk of uncertain results in CV sampling necessitating a second invasive procedure; 2) there is greater risk of mixed sampling leading to cross contamination in CV sampling; (3) there is a better performance of SEL RED after CV sampling.
The simultaneous cytogenetic investigation of both STC and LTC villi preparations will not only reduce the chance of uncertain results and follow-up investigation in AF but also improve karyotyping quality (as in singleton pregnancies)(Los et al.,1998). The identification of potential cross contamination by additional DNA analysis will eliminate the chance of misdiagnosis leading to false-negative biochemical results.

Together with the benefit of an earlier and safer SEL RED after discordant results in CV, we consider CV sampling the method of choice for prenatal diagnosis in twin pregnancies.

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Chapter 5

Diagnostic performance in AF-cells and chorionic villi

THE DIAGNOSTIC PERFORMANCE OF CYTOGENETIC INVESTIGATION IN AMNIOTIC FLUID CELLS AND CHORIONIC VILLI.

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Summary

First trimester chorionic villus sampling has not reached the popularity of 2nd trimester amniocentesis in prenatal cytogenetic diagnosis, in contrast to initial expectations. We investigated whether a difference in the diagnostic performances of cytogenetic investigation in amniotic fluid (AF) cells and chorionic villi in favour of AF-cells might justify this. Diagnostic performance was measured as laboratory failure rate, karyotype quality (G-band score, rate of follow-up samples, rate of wrong diagnoses) and karyotype representativity (rate of follow-up samples, rate of wrong diagnoses).

From 1993 - 1999, 11 883 AF-samples were investigated (AF-cells). In chorionic villi, short term culture preparations solely were karyotyped from 1993 - 1996 (n=3499) (STC-villi), short and long-term culture preparations simultaneously, provided a sufficient amount of tissue being available, from 1997 onwards (n=1829) ((STC + LTC)-villi). Laboratory failure rates were the same after amniocentesis (0.40%) and chorionic villus sampling (0.50%). G-band scores (mean ± SD) were equal in AF-cells (373 ± 38.1) and LTC-villi (364 ± 32.6) but significantly lower in STC-villi (311 ± 34.6) ($p = 0.001$). Follow-up sampling rates because of quality reasons were the same in AF-cells (0.14%), STC-villi (0.13%) and (STC + LTC)-villi (0.11%). Two wrong diagnoses turned up among AF-cells. Follow-up sampling rates because of representativity reasons differed significantly between AF-cells (0.10%), (STC + LTC)-villi (1.31%) and STC-villi (1.99%)($p < 0.001$). However, the ratios of all follow-up samples and uncertain or abnormal cytogenetic results in STC and (STC + LTC)-villi at cytogenetic risks ≥3% (0.132 and 0.160, respectively) were equal to that in AF-cells at risks < 3% (0.155). Two wrong diagnoses were made in STC-villi.

Diagnostic performance improved in the rank order of STC-villi, (STC + LTC)-villi and AF-cells. At cytogenetic risks ≥ 3%, (STC + LTC)-villi showed a diagnostic performance equal to that in AF-cells. This might justify a selective use of chorionic villus sampling.

Introduction

For prenatal laboratory investigations, pregnant women can choose between amniocentesis at 16 to 18 weeks' gestation or chorionic villus sampling at 10.5 to 13 weeks' gestation. After a successful period of first trimester cytogenetic, biochemical and DNA-investigations in chorionic villi, its use for cytogenetics has become restricted and even declined from the early ninety's onwards (Lilford, 1991; Los et al., 1998a). Theoretically, three factors could be responsible for this phenomenon. Two of these are associated with the sampling procedure; the possibly higher abortion risk after chorionic villus sampling than after amniocentesis (Rhoads et al., 1989; MRC Working Party on the Evaluation of Chorionic Villus Sampling, 1991; Smidt-Jensen et al., 1992; Lippman et al., 1992) and the potential induction of vascular disruptive syndromes by chorionic villus sampling (Firth, 1997; Froster & Jackson, 1996; Los et al., 1996; WHO/PAHO Consultation on CVS, 1999). The third factor is the lower accuracy and reliability of cytogenetic laboratory results in chorionic villi than in amniotic fluid (AF) cells; this concerns especially short term culture (STC) villi (trophoblast cells) and to a lesser extent also long term culture (LTC) villi (cultured cells of the mesenchymal villus core) (Kalousek and Dill, 1983; Wolstenholme, 1996; Ledbetter et al., 1992; Pittalis et al., 1994; Hahnemann and Vejerslev, 1997).

We evaluated this limited accuracy and reliability in detail by establishing the diagnostic performances of cytogenetic investigation in AF-cells, in STC-villi and in simultaneously investigated (STC + LTC) -villi during the years 1993 - 1999 in our cytogenetic laboratory. The diagnostic performance was assumed to consist of three components; laboratory failure rate (proportion of appropriate samples without laboratory results), karyotype quality (banding quality, proportion of follow-up sampling needed to reach sufficient quality and number of wrong diagnoses) and karyotype representativity (proportion of follow-up sampling needed to reach certainty that the established karyotype represented that of the fetus and the number of wrong diagnoses).

Materials and Methods

Samples

During the years 1993 - 1999, we received 11 935 AF-samples and 5368 chorionic villus samples in our department for prenatal cytogenetic investigation. The samples came from the University Hospital Dijkzigt (Rotterdam, The Netherlands), from the Albert Schweitzer Hospital (Dordrecht, The Netherlands) and some samples from other hospitals in The Netherlands or from abroad. Chorionic villus sampling, amniocentesis and cordocentesis were performed under continuous ultrasound guidance according to standard procedures (Holzgreve et al., 1999). Prenatal cytogenetic investigation was requested on the following indications; advanced maternal age (≥36 years; MA 36-39 or MA ≥40), fetal abnormalities on ultrasound (US-abn), parental carriership of structural chromosomal rearrangements or ESACs (extra structural abnormal

chromosomes) (Carrier), (recurrence) risk of chromosomal abnormality, previous child with multiple congenital abnormalities, family history of chromosomal abnormality, risk for X-linked diseases without further DNA or biochemical investigation (Other), prenatal biochemical and DNA investigations with additionally requested cytogenetic analysis (DNA/BIO), (recurrence) risk for neural tube defects (NTD) and follow-up investigation after uncertain results in a previous chorionic villus sample (Follow-up CV) or AF-sample (Follow-up AF).

Laboratory investigations

AF-cells were cultured by the *in situ* method, initially on glass coverslips (until early 1997) and afterwards in Labtek ® II chamber slides ™ (Nalge Nunc Int., Naperville, IL, USA). STC-villi slides were prepared by fluorodeoxyuridine synchronization (Gibas et al., 1987). LTC-villi were cultured after trypsin-EDTA and collagenase treatment of the villus material (Smidt-Jensen et al., 1989). The same Labtek ® II chamber slides ™ were used and the metaphases were harvested *in situ*. Karyotyping of AF-cells, STC-villi and LTC-villi was routinely performed by G-banding using the Pancreatine-Trypsin-Giemsa technique. Sometimes additional staining techniques were applicated; DA/DAPI-, Ag-NOR staining, endonuclease banding, C-banding or R-banding. The quality of routine banding (G-banding) of metaphases according to the International System for Human Cytogenetic Nomenclature (ISCN)(1995) was assessed with the EQAS chromosome G-band score (United Kingdom External Quality Assessment Scheme, 1988).

In AF-cell cultures, 16 cells from 16 colonies from at least two independent culture dishes were analysed routinely, according to the guidelines of The Association of Cytogenetic Technologists (ACT) Task Force (Knutsen et al., 1989). An investigation was considered as a laboratory failure when less than six colonies could be analysed or the quality of chromosome banding was insufficient. Mosaicism and pseudomosaicism (PSM) were interpreted as previously described (Boué et al., 1979). In the case of suspected PSM, more colonies were analysed according to internationally accepted criteria (Hsu et al., 1992). An investigation was repeated in a follow-up sample because of quality reasons when these criteria could not be met. An investigation was repeated because of representativity reasons when the representation of the fetal karyotype was considered uncertain; as example might figure the finding of 46,XX, add(21)(p11) [8]/46,XX [40] in AF-cells (not a PSM case according to the definition), which was, however, not recovered in fetal blood (FB)(46,XX [50]).

From chorionic villi, only STC-villi preparations were made from 1993 - 1996 (STC-villi). From 1997 onwards, STC and LTC-villi preparations were made simultaneously when 20 mg or more villus tissue was available for cytogenetic investigation ((STC + LTC)-villi). In STC-villi, the routine analysis comprised the investigation of 16 cells. When STC and LTC-villi preparations were both available, eight cells in each were investigated. Also for chorionic villi, the guidelines of the ACT Task Force for chromosome analysis were followed (Knutsen et al., 1989). An investigation was considered as a laboratory failure when less than six cells could be analysed or chromosome morphology turned out to be insufficient. An investigation was repeated

because of quality reasons when doubt remained on the structure of one or more chromosomes despite further acceptable morphology. Cytogenetic results could be normal, generalized homogeneous abnormal, generalized mosaic abnormal, or represent confined placental mosaicism (CPM) (Wolstenholme, 1996; Ledbetter et al., 1992; Pittalis et al., 1994). CPM was defined as CPM I, II and III, respectively, when a (mosaic) abnormal karyotype in STC-villi, LTC-villi, or both was accompanied by a normal karyotype in the fetus or amniotic fluid (Wolstenholme, 1996). In the case of CPM without the analysis of LTC-villi, it was designated CPM I or III. Our management of uncertain cytogenetic results has been described in detail for STC-villi and (STC + LTC)-villi (Los et al., 1998a; Van den Berg et al., 2000). All follow-up investigations for the discrimination of generalised abnormalities from CPM-cases were carried out because of representativity reasons.

Cytogenetic investigation regularly included the use of fluorescence *in situ* hybridization (FISH); the methods, applications and results have been previously reported (Los et al., 1998a; Van den Berg et al., 1999, 2000; Van Opstal et al., 1993, 1998).

Sometimes additional or follow-up investigations were needed before a definite prenatal cytogenetic result and its interpretation could be reported. Additional investigations involved parental karyotyping in the case of a structural rearrangement or (mosaicism of) an ESAC and ultrasound (US) investigation for the establishment of potential phenotypic effects in the case of uncertain laboratory results or uncertainty about clinical effects of otherwise certain laboratory results. Follow-up investigations comprised second or third prenatal invasive procedures for repeat cytogenetic analysis. In multiple pregnancies, a repeat sample of each fetus was taken.

Statistical analysis

Statistical analysis comprised χ^2 tests (of proportions), the establishment of 95% confidence intervals (95% CIs) of ratios and analysis of variances (ANOVA).

Results

Cytogenetic results were reported in 11 883 of 11 935 AF-samples (99.6%) and in 5328 of 5368 chorionic villus samples (99.3%). No cytogenetic results could be reported in the remaining samples due to laboratory failure or to inappropriate quality or quantity of the sample (Table 1). The laboratory failure rates of cytogenetic investigation in AF-cells, STC-villi and (STC + LTC)-villi were equal (0.40%, 0.48% and 0.54%, respectively).

An example of the chromosome quality in our preparations of STC-villi, LTC-villi and AF-cells is shown in a case in which all three compartments were prenatally karyotyped because of a mosaic abnormality in STC-villi (Figure 1). The chromosome G-band score (mean ± SD) was 311 ± 34.6 in STC-villi, 364 ± 32.6 in LTC-villi and 373 ± 38.1 in AF-cells (ANOVA, $F = 9.08$, $df_1 = 2$, $df_2 = 27$; $p = 0.001$), representing a significantly lower banding quality in STC-villi.

Table 1 - Numbers of received samples, reported and failed cytogenetic investigations during the years 1993 - 1999; reasons for failure and calculation of laboratory failure rate

Condition	Amniotic Fluid (1993 - 1999)	STC-villi (1993 - 1996)	(STC + LTC)-villi (1997 - 1999)
Received samples	11 935	3522	1846
Cytogenetic results reported	11 883	3499	1829
Laboratory failure of appropriate sample			
- growth, harvest failure	29	13	9
- infection	3	-	-
- karyotype quality failure	16	4	1
Sampling failure			
- insufficient quantity of sample	3	3	4
- insufficient quality of sample	1	3	1
Transport failure			
- leakage, infection	-	-	1
- exceeding maximal time for transport	-	-	1
Total failure	52	23	17
Sampling repeated	34 (0.28%)	19 (0.54%)	9 (0.49%)
Laboratory failure rate	$\frac{(29 + 3 + 16)}{(11\,935 - 4)}$	$\frac{(13 + 4)}{(3522 - 6)}$	$\frac{(9 + 1)}{(1846 - 7)}$
	0.40%	0.48%	0.54%
			NS

Furthermore, chromosomes in AF-cells and LTC-villi showed a similar morphology with a more or less sharp banding pattern, whilst those in STC-villi displayed generally a certain fuzzyness.

The number of normal and abnormal karyotypes, carriers, CPM cases, PSM cases, and "other" cytogenetic results among the various indications are shown in the Tables 2 - 4 for AF-cells, STC-villi and (STC + LTC)-villi, respectively.
The "other" cytogenetic results include mosaic or discrepant findings due to maternal cell contamination (MCC), cross contamination (admixing of the sample of fetus I to that of fetus II and/or vice versa, in a twin pregnancy), wrong sampling (sampling one fetus twice and the other not at all in a twin pregnancy) and chimaeric findings.

The numbers of additional and follow-up investigations, necessary to reach the final cytogenetic results and subsequent clinical interpretations, are shown in the Tables 2 - 4. The frequencies of parental karyotyping in the case of unexpected structural chromosome abnormalities or ESACs did not differ significantly between AF-cells, STC-villi and (STC + LTC)-villi. In contrast to this, the difference between the frequencies of US-investigations was highly significant ($\chi^2 = 94.72$, df = 2; $p < 0.001$). The reasons for follow-up sampling are given in Table 5. The rates of follow-up sampling because of quality reasons were equal in AF-cells, STC-villi and (STC + LTC)-villi.

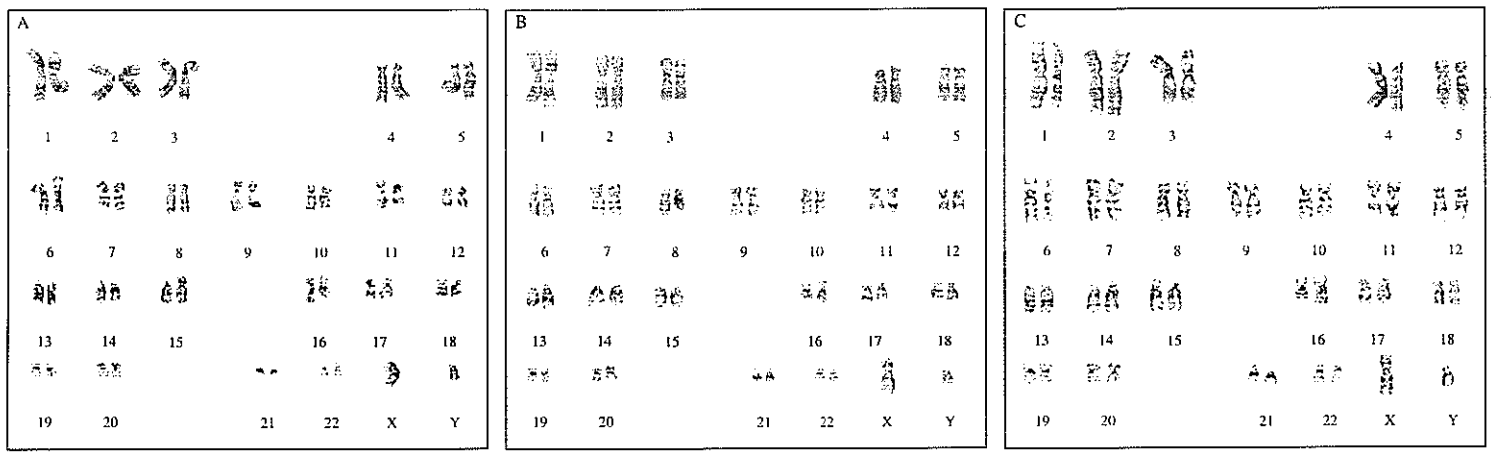


Figure 1 - Karyotypes of the same pregnancy in **(A)** STC-villi displaying 318 bands (average quality), **(B)** LTC-villi displaying 325 bands (minus one SD of mean quality and **(C)** AF-cells displaying 377 bands (average quality), according to the International System for Human Cytogenetic Nomenclature (ISCN).[27]

Table 2 - Amniotic Fluid 1993-1999 (AF-cells)

							Additional investigation		Follow-up investigation			
							Parental		2nd sample		3rd sample	
Indication	N	Normal	Abnormal	Carrier	PSM	Other	karyotype	US	AF	FB	AF	FB
MA 36-39	6285	6024	74	34	144	9[1]	43	19	13	1[2]	-	-
MA ≥40	1011	949	33	5	24	-	5	5	5	-	-	-
US-abn	1097	925	140	8	23	1[3]	26	7	1	-	-	-
Carrier	65	32	2	29	2	-	-	1	-	-	-	-
DNA/BIO	153	148	-	2	3	-	2	-	-	-	-	-
NTD	1061	1020	9	4	27	1[3]	4	4	4	-	-	-
Other	2081	2002	30	10	36	3[3]	14	5	2	-	-	-
Total (N_1)	11 753	11 100	288	92	259	14	94	41	25	1	-	-
Follow-up CV	105	80	18	5	2	-	1	14	-	-	-	2[4]
Follow-up AF	25[5]	17	7	1	-	-	-	4	-	-	-	3[6]
All (N_2)	11 883	11 197	313	98	261	14	95	59	25	1	-	5

1 = MCC (7 cases), 1 culture dish 46,XX, the other 3 dishes 46,XY (1 case); discrepancy between AF and US concerning fetal gender of one fetus of twin pregnancy (1 case)
2 = FB; normal
3 = MCC-cases
4 = FB; carrier (2 cases)
5 = follow-up sample because of discrepancy of fetal gender at ultrasound (♂) and AF karyotype (46,XX), due to mixing up of samples (1 case)
6 = FB; abnormal (2cases), normal (1case)

Table 3 - Chorionic villi 1993-1996; analysis of STC-villi only (STC-villi)

						Additional investigation		Follow-up investigation	
Indication	N	Normal	Abnormal	Carrier	CPM I or III	Parental karyotype	US	AF	FB
MA 36-39	2282	2191	33	7	51	21	42	54	-
MA ≥40	447	424	14	1	8	2	7	6	-
US-abn	165	116	46	-	3	1	3	4	-
Carrier	80	41	9	30	-	-	-	-	-
DNA/BIO	206	201	2	-	3	1	2	3	-
Other	319	307	6	-	6	2	9	9	-
All	3499	3280	110	38	71	27	63	76	-

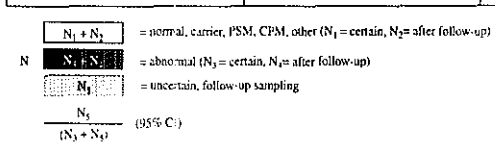
The rates of follow-up sampling because of representativity reasons and, hence, the total follow-up sampling rates showed highly significant differences in the rank order AF-cells, (STC + LTC)-villi and STC-villi (Table 5).

The ratios and their 95% CIs of the numbers of all follow-up samples (for quality, representativity and other reasons) and the numbers of abnormal or uncertain results at cytogenetic risks < 3%, ≥3% and at all cytogenetic risks are shown in Figure 2A. At all risks, there is a significant difference between these ratios in the rank order AF-cells, (STC + LTC)-villi and STC-villi (Figure 2B). However, the ratios in STC and (STC + LTC)-villi at cytogenetic risks ≥ 3% did not differ from that in AF-cells at cytogenetic risks < 3% (Figure 2B).

During the study period, a wrong prenatal cytogenetic diagnosis was made twice in STC-villi; both false-positive cases are examples of the limited representativity of the karyotype in chorionic villi for that in the fetus (Table 6). In AF-cells, a wrong diagnosis was made also in two instances; the first case is an example of the limitation of karyotype quality in AF-cells compared to blood lymphocytes and the second case of the limitations of FISH investigation (Table 6).

A

Sample	Cytogenetic risk < 3% (MA 36-39, DNA/BIO, NTD, other)	Cytogenetic risk ≥ 3% (MA ≥ 40, US-abn, carrier)	All cytogenetic risks
Amniotic fluid	9580 → 9451 + 16; 109 + 4; 20 0.155 (0.093 - 0.217)	2173 → 1995 + 3; 172 + 3; 6 0.034 (0.012 - 0.072)	11753 → 11446 + 19; 281 + 7; 26 0.085 (0.056 - 0.122)
STC-villi	2807 → 2713 + 53; 28 + 13; 66 0.702 (0.599 - 0.792)	692 → 616 + 7; 66 + 3; 10 0.132 (0.065 - 0.229)	3499 → 3329 + 60; 94 + 16; 76 0.447 (0.372 - 0.522)
(STC + LTC)-villi	1321 → 1293 + 16; 12 + 0; 16 0.571 (0.372 - 0.755)	508 → 427 + 11; 68 + 2; 13 0.160 (0.088 - 0.259)	1829 → 1720 + 27; 80 + 2; 29 0.266 (0.183 - 0.349)



B

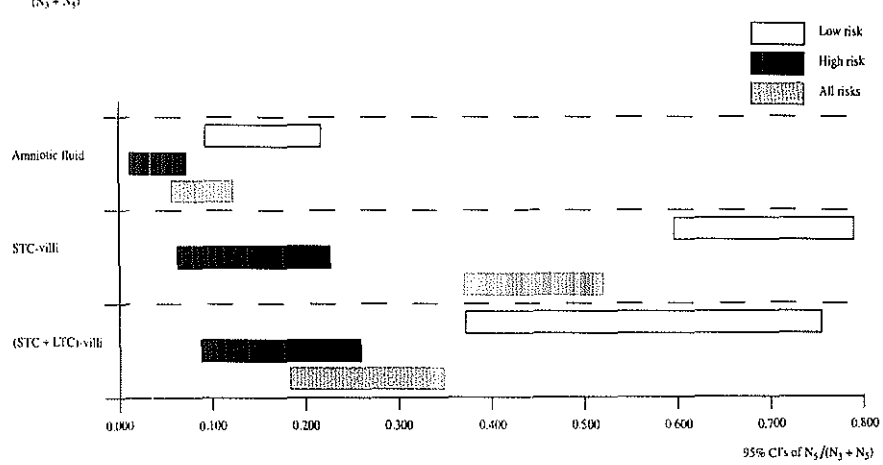


Figure 2 - (A) Numbers of normal, abnormal, and uncertain cytogenetics results in AF-samples, STC-villi, and (STC + LTC)- villi at low (<3%), high (≥ 3%) and all cytogenetic risks. Furthermore, the number of repeat investigations (only second samples) with finally normal or abnormal results are shown. The ratios of follow-up samples and abnormal or uncertain results with their 95% CI's are indicated. (B) An overview of the various 95% CI's of the three types of samples at the different cytogenetic risks.

Table 4 -Chorionic villi 1997 - 1999; analysis of STC-villi only in 290 cases (16%) and of (STC + LTC)- villi in 1539 cases (84%)

		Normal				CPM					Additional investigation		Follow-up investigation	
Indication	N	2N	4N/2N	Abn	Carrier	I	II	III	I or III	Other	Parental karyotype	US	AF	FB
MA 36-39	954	852	63	11	6	11	5	1	3	2[1]	9	16	13	-
MA ≥40	297	258	16	11	1	3	4	1	1	2[2]	4	8	9	-
US abn	157	90	6	56	2	1	1	1	-	-	5	1	-	-
Carrier	54	22	4	3	22	1	2	-	-	-	-	3	4	(2[3])
DNA/BIO	170	160	7	-	-	1	1[4]	-	1	-	-	-	2	-
Other	197	173	18	1	2	3	-	-	-	-	2	3	1	-
All	1829	1555	114	82	33	20	13	3	5	4	20	31	29	(2)

2N = diploidy, 4N/2N = tetraploidy/diploidy mosaicism; 1 = cross contamination in one fetus of twin pregnancy (one case); MCC (one case); 2 = wrong sampling; one fetus twice and the other not at all (one case); MCC (one case); 3 = amniocentesis and fetal blood sampling performed simultaneously; AF-results assumed not to be 100% representative. Therefore, FB is considered as a follow-up (third) sample of the second AF-sample and placed between brackets here (two samples); 4 = also MCC

Table 6 -Wrong prenatal cytogenetic diagnoses in the period 1993 - 1999 among 11 883 AF-samples and 5328 chorionic villus samples

Prenatal sample	Indication	Prenatal karyotype	Pregnancy - outcome	Postnatal karyotype or clinical findings
STC - villi (N = 3499)	MA 36-39	46,XY, der(10) de-novo	TOP	46,XY in fetal fibroblasts
	MA 36-39	47,XY,+8[3]/46,XY[15]	TOP against our advise	46,XY in fetal fibroblasts
(STC + LTC)- villi (N = 1829)	-	-	-	-
AF-cells (N = 11 883)	Other	46,XX	Normal birth, developmental delay, various congenital malformations	46,XX, del(18)(q22.2qter) de-novo in lymphocytes
	NTD	45,X[11]/46,X,+ mar [8] FISH; ESAC≠X or Y chromosomal material	Continuation of pregnancy, despite counselling a potentially severe abnormal phenotype	mild Turner phenotype FISH[1]; ESAC = Xp material

der = derivative chromosome, TOP = termination of pregnancy; 1 = FISH investigation on the original amniotic fluid cell metaphases

Table 5 - Reasons for follow-up cytogenetic investigation in second (and third) samples and the rates of follow-up sampling for quality and for representativity reason

Reason for follow-up sampling	Amniotic Fluid (1993 - 1999) N_1 = 11 753 N_2 = 11 883		STC-villi (1993 - 1996) N = 3499		(STC + LTC)-villi (1997 - 1999) N = 1829	
	AF	FB	AF	FB	AF	FB
2nd sample						
- quality reasons	15	-	4	-	2	-
- representativity reasons	5	1	69	-	24	-
- both quality and representativity	3	-	1	-	-	-
- other reasons	2[1]	-	2[2]	-	3[3]	-
3rd sample						
- representativity reasons	-	4	-	-	-	(1)[5]
- other reasons	-	1[4]	-	-	-	(1)
All	25	6	76	-	29	(2)
Follow-up sampling rate - for quality reasons	$\frac{15 + (3 \times 0.5)}{11\,753\ (N_1)}$		$\frac{4 + (1 \times 0.5)}{3499}$		$\frac{2}{1829}$	
	0.14%		0.13%		0.11%	NS
- for representativity reasons	$\frac{6 + (3 \times 0.5)}{11\,753\ (N_1)} + \frac{4}{11\,883\ (N_2)}$		$\frac{69 + (1 \times 0.5)}{3499}$		$\frac{24}{1829}$	
	0.10%		1.99%		1.31%	
					$\chi^2 = 176.25$, df = 2; $p < 0.001$	
Total follow-up sampling rate	$\frac{26}{11\,753\ (N_1)} + \frac{5}{11\,883\ (N_2)}$		$\frac{76}{3499}$		$\frac{29}{1829}$	
	0.26%		2.17%		1.59%	
					$\chi^2 = 146.51$, df = 2; $p < 0.001$	

1 = discrepancy of fetal sex between AF results and ultrasound in one fetus of a twin pregnancy (due to wrong sampling), second sample of both fetuses

2 = second samples of fetuses with normal karyotypes in two twin pregnancies

3 = wrong sampling (one fetus twice (twice abnormal), the other not sampled at all) leading to two second samples, one for quality reasons and one for other reasons; second sample of fetus with normal karyotype in twin pregnancy (1 case); X-inactivation studies in case of a (X;autosome) translocation (1 case)

4 = X-inactivation studies in case of a (X;autosome) translocation (1case)

5 = third samples are considered to be follow-up samples of the second rather than of the first samples and, therefore, the numbers placed between brackets at the first samples

Discussion

For over 20 years, prenatal diagnosis in AF-cells has been regarded as reliable and accurate (Golbus et al., 1979). Some time after the introduction of chorionic villus sampling, amniocentesis and laboratory investigations in AF-cells became the "gold standard" of invasive prenatal genetic diagnosis (Stranc et al., 1997). The reliability and accuracy of cytogenetic laboratory results of chorionic villi have been subject to many investigations. However, a restricted accuracy and reliability of cytogenetic results in chorionic villi compared to AF-cells did not influence women in choosing amniocentesis or chorionic villus sampling, in contrast to professionals in the field of prenatal diagnosis (Heckerling et al., 1994; Kuppermann et al., 1999).

In the literature, laboratory failure rates after amniocentesis have been reported between 0.1% and 0.4% (Lippman et al., 1992; Smidt-Jensen et al., 1993; Waters and Waters, 1999). These figures range from 0.3% to 2.0% after chorionic villus sampling (Lippman et al., 1992; Ledbetter et al., 1992; Smidt-Jensen et al., 1993; Waters and Waters, 1999). Our figure for AF-cells (0.40%) is just at the reported upper-limit and those for chorionic villi (0.48% for STC-villi, 0.54% for (STC + LTC)-villi) are in the lower region of the reported range. However, our definition of laboratory failure rate might be rather more tight than the published failure rates. The laboratory failure rates in AF-cells and chorionic villi as parameter of the diagnostic performance of cytogenetic investigation did not differ significantly from each other in our laboratory.

Prenatal cytogenetic diagnosis requires a resolution of 400 bands per haploid set and a minimum of 150 bands in direct chorionic villi preparations (United Kingdom External Quality Assessment Scheme, 1988). Although STC-villi preparations show a much better chromosome banding than direct villi slides, the G-band score in STC-villi was significantly less than in LTC-villi or AF-cells. Theoretically, this inferior karyotype quality is expected to result in a higher rate of follow-up investigations and in more wrong (missed or erroneously assigned) diagnoses. However, this was not substantiated by the figures on these parameters, that turned out to be equal in STC-villi, (STC + LTC)-villi and AF-cells. The only two wrong diagnoses were actually made in AF-cells. The literature on wrong prenatal cytogenetic diagnoses due to limitations in karyotype quality is scarce; for chorionic villi, figures have been reported between 3/7415 (0.04%) (ACC Working Party on Chorionic Villi in Prenatal Diagnosis, 1994), 1/11 436 (0.009%) (Ledbetter et al., 1992) and 4/62 865 (0.006%) (Hahnemann & Vejerslev, 1997); for AF-cells only some sporadic cases have been documented (Golbus et al., 1979; Winsor et al., 1999). Karyotype quality as parameter of the diagnostic performance of cytogenetic investigation turned out to be equal in AF-cells and (STC + LTC)-villi, but to be less in STC-villi, albeit without noticable consequences in our study.

The allocation of normal and abnormal cells from the 8 to 16-cell stage to the embryonic compartments of the fetus proper, extra embryonic mesoderm (EEM) and trophoblast in the case of arising of mosaicism in the early embryo may lead to unequal distributions in these compartments (Wolstenholme, 1996; Pittalis et al., 1994; Los et al., 1998b). The investigation of the compartment of interest, that of the fetus proper, in AF-cells and of the other compartments, those of the trophoblast and EEM, in STC- and

LTC-villi, respectively, implicates a fundamental biological difference between AF-cells and chorionic villi in favour of AF-cells concerning karyotype representativity. The significant differences in the frequencies of additional US-investigations and in the follow-up sampling rates because of representativity reasons in AF-cells, STC-villi and (STC + LTC)-villi are in agreement with this. In the literature, no figures for the follow-up sampling rates just because of representativity reasons could be found, only rates of follow-up sampling for all cytogenetic (quality and representativity) reasons. Figures have been reported between 0.3% and 0.4% for AF-cells (Smidt-Jensen et al., 1993; Winsor et al., 1999), between 2.0% and 2.2% for chorionic villi with the analysis of STC-preparations only (Los et al., 1998a; Leschot et al., 1996), between 0.8% and 1.8% with the analysis of LTC-preparations only (Sundberg et al.,1999; Sikkema-Raddatz et al., 2000) and between 0.9% and 1.6% with the simultaneous analysis of STC- and LTC-preparations when a sufficient amount of villus material was available (Lippman et al., 1992; Ledbetter et al., 1992; Smidt-Jensen et al., 1993). Our figures of total follow-up sampling rates in AF-cells (0.26%), (STC + LTC)-villi (1.59%) and STC-villi (2.17%) were within these ranges. Two wrong (false-positive) diagnoses due to limitations of karyotype representativity were made in STC-villi. Karyotype representativity as parameter of the diagnostic performance of cytogenetic investigation declined significantly in the rank order AF-cells, (STC + LTC)-villi and STC-villi. However, when the numbers of follow-up samples were considered in relation to the numbers of abnormal or uncertain results, rather than the numbers of all investigations, the calculated ratios turned out to be equal for chorionic villi at cytogenetic risks ≥ 3% and AF-cells at cytogenetic risks < 3%.

The diagnostic performances of cytogenetic investigation in the studied prenatal samples increasewith considerable intervals in the rankorder of STC-villi, (STC + LTC)-villi and AF-cells, which justify a restricted use of chorionic villus sampling.
However, at cytogenetic risks ≥3%, the diagnostic performance of cytogenetic investigation in (STC + LTC)-villi, equals the "gold standard" of prenatal diagnosis in AF-cells, which might justify a selective rather than a restricted use of chorionic villus sampling.

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Chapter 6

Specific and/or recurrent problems

Chapter 6.1

Trisomy 9

PRENATAL DIAGNOSIS OF TRISOMY 9: CYTOGENETIC, FISH AND DNA STUDIES.

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Summary

A cytogenetic survey and follow-up studies were performed in eight cases of full, mosaic, and pseudomosaic trisomy 9 prenatally diagnosed among 36.213 prenatal samples in our department between August 1970 and July 1996. Besides conventional chromosome analysis, interphase fluorescent *in situ* hybridization (FISH) was employed. FISH turned out to be a rapid and accurate method for verification of trisomy cell lines and could provide additional information to the prenatal cytogenetic results. FISH also enables the study of uncultured specimens of amniotic fluid, not accessible for traditional cytogenetic analysis. In three cases, retrospective DNA analysis showed the supernumerary chromosome 9 to be of maternal origin. The disomic cell lines in both mosaic trisomy 9 cases showed maternal uniparental disomy.

Introduction

Prenatal diagnosis of trisomy 9, in a mosaic or a non-mosaic state, is an uncommon chromosome abnormality, first described by Francke et al. (1975) and Pfeiffer et al. (1984). The prenatal detection of trisomy 9 mosaicism in amniotic fluid (AF) cells has posed a serious problem for genetic counselling and appropriate monitoring of the pregnancy, since in some cases the abnormality could not be confirmed, probably due to limitation of the trisomic cell line to the fetal membranes (Hsu and Perlis, 1984; Pfeiffer et al., 1984). Trisomy 9 (mosaicism) observed in chorionic villi (CV) also poses a serious dilemma because it is an unusual trisomy (Ledbetter et al.,1992) and may reflect generalized mosaicism as well as mosaicism confined to the placenta (CPM) (Kalousek and Dill, 1983; Kalousek et al., 1987, 1991; Appelman et al., 1991).

Differentiation between pseudomosaicism and true mosaicism or CPM and generalized mosaicism is often difficult, due to time constraints and a limited amount of available metaphases. Fluorescence *in situ* hybridization (FISH) allows the rapid detection of numerical chromosome aberrations in a large number of cultured as well as uncultured cells.

In this study, eight cases of pseudomosaic, mosaic or non-mosaic trisomy 9 are presented; six cases were detected in AF and two cases diagnosed in CV. We have applied FISH with the chromosome 9-specific probe pHUR98 (Moyzis et al., 1987) to study (1) AF cultures previously classified as either pseudo- or true mosaic, in order to determine whether FISH could provide additional information for the differentiation of pseudomosaicism from true mosaicism, and (2) CV preparations and uncultured AF specimens, in order to differentiate CPM from generalized mosaicism. DNA analysis was performed on AF cells, placental tissue, and parental lymphocytes to establish the parent of origin of the supernumerary chromosome 9 and to identify the missing copy in the disomic cell line of the mosaic cases.

Materials and Methods

Patients

Between August 1970 and July 1996, a total of 36.213 prenatal cytogenetic investigations were carried out in our department. For this study, we selected all cases with a numerical chromosome 9 abnormality. This resulted in six cases among the amniotic fluid samples and two cases among the chorionic villus samples with either a full trisomy 9, a mosaic trisomy 9, a pseudomosaic, or a confined placental mosaicism (CPM) for trisomy 9.

Cytogenetic investigations

Transabdominal chorionic villus sampling (TACVS), amniocentesis, and cordocentesis were performed under ultrasound guidance. CV were incubated overnight using fluorodeoxyuridine (FdU) synchronization (Gibas et al.,1987).
AF cells were cultured by the *in situ* method on glass coverslips. Metaphase spreads of fetal lymphocytes and skin fibroblasts were prepared according to standard techniques. Trypsin-Giemsa staining was routinely used for karyotyping and a minimum of 16 cells/colonies were analysed in each case. In cases of chromosome mosaicism, pseudomosaicism, or when CPM was suspected, more cells were analysed and follow-up investigations were suggested. Mosaicism and pseudomosaicism were interpreted according to definitions of Boué et al. (1979) and Hsu and Perlis (1984).

Fluorescence in situ *hybridization (FISH)*

Interphase FISH was performed on semi-direct CV preparations, cultured amniocytes, fetal lymphocytes, and fetal skin fibroblasts according to standard protocols, as well as on slides of uncultured amniocytes (Van Opstal et al.,1993) and fetal lymphocytes. The latter were prepared by incubating the cells in 0.075M KCl at 37°C for 20 min. Subsequently the cells were fixed by three changes of methanol-acetic acid (3:1) and dropped onto slides (Garnham and Sutherland,1987).

The chromosome 9-specific probe pHUR98 (Moyzis et al.,1987) was used for detection of the chromosome 9 copy number in interphase nuclei. In one case the chromosome 7-specific probe pα7t1 (Waye et al.,1987) was also used, for detection of the chromosome 7 copy number.

Probes were labelled by nick translation using either biotin-11-dUTP (BRL), biotin-16-dUTP or digoxigenin(DIG)-11-dUTP (Boehringer Mannheim).

The probes pHUR98 and pα7t1 (40 ng in 10 µl of 60 percent formamide/2xSSC) and target DNA were denaturated simultaneously for 3 min at 80°C. Hybridization was performed overnight at 37°C. Slides were washed three times in 50 per cent formamide/2xSSC at 42°C for 5 min, followed by three changes of 2xSSC, twice at 42°C and once at 60°C.

Probe detection of biotinylated probes was achieved via alternating application of fluoresceinated avidin and biotinylated anti-avidin antibody (Vector Laboratories). DIG-labelled probes were detected with one layer of anti-DIG-fluorescein isothiocyanate (FITC)(Boehringer Mannheim). Finally, the slides were mounted in an antifade solution containing propidium iodide and DAPI as a counterstain.

Slides were examined under a Leica Aristoplan epifluorescence equipped microscope and images were captured with the Genetiscan ProbeMaster system (Perceptive Scientific International Ltd) including a Xybion cooled CCD 24-bit colour camera.

For each sample, a minimum of 100 intact non-overlapping and non-clumped interphase nuclei were counted. Nuclei without signals were not included in the

data. For the interpretation of the FISH results the 95% confidence interval of the upper reference limit for the proportion of cells with three signals was established in diploid tissue control samples, according to Lomax et al. (1994).

DNA analysis

In three cases, molecular studies were performed on DNA isolated from uncultured and cultured amniocytes (passage 2 or more), cultured placental tissue, and parental lymphocytes according to standard techniques. Studies on the parent of origin of the additional chromosome 9 were performed using polymerase chain reaction (PCR) amplification of polymorphic microsatellite markers. The chromo-some 9-specific loci D9S147E (9pter and 9qter), D9S158 (9q34.3), D9S171 (9p21), D9S175 (9q13-q21), D9S118 (9q31), and D9S156 (9p23) were studied (Reed et al.,1994).

The detection was performed with fluorescently labelled primers by semi-automated methods using an ABI prism 377 DNA sequencer as described previously by Reed et al. (1994). Conclusions about parental origin required at least two informative markers.

Results

Between 1970 and mid-1996, 25.073 diagnostic AF samples and from September 1983 to July 1996, 11.140 diagnostic CV samples were received in our laboratory for chromosome analysis. A total of eight cases with a numerical chromosome 9 abnormality were found among the AF and CV groups. The prenatal cytogenetic and FISH studies are summarized in Table 1. Ultrasound investigations, pregnancy-outcome and confirmatory studies are shown in Table 2.

Amniotic fluid group

A total of six cases with a numerical chromosome 9 abnormality were found among this group. A full trisomy 9 was seen twice (cases 1 and 2). Both pregnancies were terminated at the parents' request. Confirmation of the trisomy 9 in fetal tissue was only possible in case 2, due to growth failure of fetal fibroblasts in case 1. Both fetuses showed the trisomy 9 phenotype as described by Chitayat et al. (1995).

A mosaic trisomy 9 occurred only once (case 3). The parents were informed of the cytogenetic results and further diagnostic procedures in order to reach definite certainty were recommended: ultrasound investigation, repeat amniocentesis, CVS and fetal blood sampling. Ultrasound examination of the fetus revealed some minor abnormalities. FISH on uncultered amniocytes of the repeat amniotic fluid, semi-direct CV preparations, and uncultured fetal lymphocytes revealed the presence of a trisomy 9 cell line in all investigated compartments and the parents chose to terminate the pregnancy. Trisomy 9 mosaicism was confirmed in amniotic and chorionic membrane and by the phenotypic appearance of the fetus.

Table 1 - Cytogenetic and FISH results in the eight prenatal cases with a numerical chromosome 9 abnormality

Case No.	First sample	Karyotype [No. of AF colonies/ CV cells]	Follow-up laboratory investigations						Prenatal diagnosis
			Sample	% of nuclei with 1-3 signals				Karyotype [No. of AF colonies/ CV,FB cells]	
				1	2	3	N		
1	AF	47,XX,+9 [16]	-						Trisomy 9
2	AF	47,XX,+9 [21]	-						Trisomy 9
3	AF	47,XY,+9[4]/46,XY[26]	AF culture	0	60	**40**	400		Mosaic trisomy 9
			AF2 direct	6	71	**24**	170		
			CV direct	0	8	**92**	200	47,XY,+9[8]	
			FB direct	4	84	**12**	202		
			FB culture	-	-	-	-	46,XY[30]	
4	AF	47,XX,+9[1]*/46,XX[18]	AF direct	5	95	0	152		Pseudomosaic trisomy 9
			AF culture[a]	5	90	5	200		Type A
5	AF	47,XY,+9[1]/46,XY[5]	AF culture[b]	4	59	**27**	189		
			AF culture[a]	4	92	4	400		Pseudomosaic trisomy 9
			AF2 direct	11	85	4	100		Type C
			AF2 culture	-	-	-	-	46,XY[17]	
			FB direct	12	80	9	207		
			FB culture	5	94	3	202	46,XY[100]	
6	AF	47,XY,+9[1]/46,XY[17]	AF culture[a]	9	88	3	436		Pseudomosaic trisomy 9 Type B
7	CV	47,XX,+9[30]	CV direct	0	4	**96**	200		Mosaic trisomy 9
			AF2 direct	0	62	**38**	250		
			AF2 culture	-	-	-	-	47,XX,+9[7]/46,XX[30]	
8	CV	48,XY,+7,+9[2]/46,XY[17]	CV direct	-	-	-	-		CPM
			AF culture	-	-	-	-	46,XY[16]	

AF =amniotic fluid; AF2 = repeat amniotic fluid; CV = chorionic villi; FB = fetal blood; CPM = confined placental mosaicism; - = not tested or non-informative results;
* = colony includes abnormal and normal cells; a = dish without trisomy 9 colonies; b = dish with trisomy 9 colonies.

Table 2 - Results of ultrasound investigation and pregnancy-outcome in the 8 cases of (mosaic) trisomy 9

Case no.	Prenatal Diagnosis	Ultrasound investigation	Pregnancy-outcome	Confirmatory cytogenetic investigation
1	Full trisomy	-	TOP: 19 weeks, ♀ fetus with severe facial malformations	Failure of fibroblast culture
2	Full trisomy	20 weeks, IUGR, skeletal malformations	TOP: 20 weeks, ♀ fetus with facial dysmorphisms and skeletal malformations	47,XX,+9 (fetal skin fibroblasts)
3	Mosaicism	19 weeks, minor abnormalities (plexus chor. cysts, micrognathia)	TOP: 20 weeks, ♂ fetus with facial dysmorphisms, no autopsy	FISH: 70% +9 (amniotic membrane), 88% +9 (chorionic membrane) failure of fibroblast culture
7	Mosaicism	16 weeks, IUGR	TOP: 16 weeks, ♀ fetus , growth retarded	FISH: 100% +9 in placental tissue, failure of fibroblast culture
4	Pseudomosaicism type A	29 weeks, IUGR	Liveborn♀, 37 weeks, SGA, transient respiratory and feeding difficulties	46,XX[100] (blood lymphocytes)
6	Pseudomosaicism type B	-	Liveborn normal ♂	-
5	Pseudomosaicism type C	Normal	Liveborn normal ♂	-
8	CPM	Normal	Liveborn normal ♂	-

TOP = termination of pregnancy; IUGR = intrauterine growth retardation; CPM = confined placental mosaicism; SGA = small for gestational age.

A pseudomosaic trisomy 9 occurred in three cases (Nos 4, 5 and 6). In case 4, amniocentesis was performed at 29 weeks' gestation because of intrauterine growth retardation (IUGR). The karyotype showed a pseudomosaic state, type A.

Because uncultured amniocytes from all patients with ultrasound abnormalities are stored in our laboratory, we could perform FISH on interphase nuclei of uncultured amniocytes which showed the absence of trisomy 9 cells. The infant was delivered at 37 weeks and had some minor and transient respiratory and feeding problems.

In case 5, only a few colonies could be examined and these were in a pseudomosaic state. FISH on interphase nuclei of the remaining (trypsinized) cultured amniocytes present on the bottom of all dishes showed a significant population of trisomy 9 cells (27%) in the dish with the trisomy 9 colony. The possibility of a real mosaicism was discussed and further diagnostic procedures were recommended, such as ultrasound examination, cordocentesis and repeat amniocentesis. Ultrasound examination at the time of the repeat amniocentesis and cordocentesis revealed no abnormalities. FISH on interphase nuclei of uncultured amniocytes and uncultured fetal lymphocytes turned out to be normal.
Chromosome analysis of both cultured tissues yielded normal karyotypes and the pregnancy continued; a normal boy was born at term.

In case 6, FISH on interphase nuclei of the remaining cells was performed and revealed no trisomy 9 cells, so further diagnostic procedures were not necessary.

Chorionic Villus group

Two cases with a numerical chromosome 9 abnormality were found among this group. In one case (No. 7), we found a full trisomy 9 and in another case (No. 8), a mosaic trisomy 9. The possibility of false-positive results was discussed and further diagnostic procedures were carried out. Depending on those results, amniocentesis and structural ultrasound examination of the fetus were recommended.

In case 7, FISH on uncultured amniocytes showed an increased percentage of interphase nuclei with three signals, indicating the presence of a mosaic trisomy 9. This was confirmed by karyotyping. The pregnancy was terminated at the parents' request. Unfortunately, confirmation of the trisomy 9 was possible only on placental tissue and amniotic and chorionic membrane.

In case 8 , FISH on semi-direct preparations turned out to be uninformative and FISH on uncultured amniocytes was not yet available. Chromosomes from cultured amniocytes revealed normal results and the pregnancy was continued.

DNA analysis

Retrospective DNA analysis was performed (after prolonged culturing of AF cells) in case 2 showing a full trisomy 9 in AF and in the cases 3 and 7 showing a mosaic trisomy 9 in AF. The results are summarized in Table 3.

Table 3 - Alleles of the informative chromosome 9 markers in the three families investigated

Case No.	Marker	Localization	Father	Mother	Placenta	Uncultured AF cells	Cultured AF cells
2	D9S118	9q31	2, 4	1, 3	-	-	1, 2, 3
	D9S156	9p23	3	1, 2	-	-	1, 2, 3
3	D9S158	9q34.3	3	1, 2	-	-	1
	D9S175	9q13-q21	1, 4	2, 3	-	-	2, 3
7	D9S158	9q34.3	1, 4	2, 3	1, 2, 3	2, 3	2, 3
	D9S171	9p21	3	1, 2	1, 2, 3	1, 2 ,3	1, 2
	D9S175	9q13-q21	2	1, 3	1, 2, 3	-	1, 3

- = not investigated; AF = amniotic fluid

In the full trisomy 9 case, the supernumerary 9 turned out to be of maternal origin, since two maternal alleles and one paternal allele were identified in cultured AF cells with two markers (Fig. 1A). In both cases with mosaicism, the cultured AF cells apparently lost the trisomic cell line and revealed maternal uniparental disomy

(UPD) and the absence of a paternal allele for chromosome 9 with two and three of the tested markers, respectively (Figs 1B and 1C).

Heterodisomy was seen in case 7 and partial isodisomy/heterodisomy in case 3. However, in case 7, cultured placental tissue and uncultured AF cells showed a normal and a weak paternal allele, respectively, in contrast to the presence of only maternal alleles in cultured amniotic fluid cells.

Discussion

Trisomy 9 (mosaicism) is an uncommon but well-known finding in prenatal

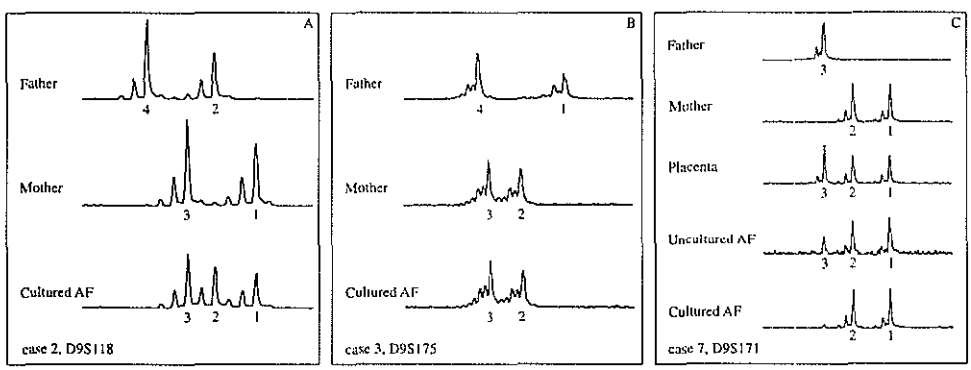

Figure 1 - PCR analysis of microsatellite markers. **(A)** Case 2. For D9S118, the cultured AF cells have both maternal alleles (1, 3) and one paternal allele (2). **(B)** Case 3. For D9S175, the cultured AF cells show only the maternal alleles (2, 3). **(C)** Case 7. For D9S171, the placenta has both maternal alleles (1, 2) and one paternal allele (3). The uncultured AF cells have both maternal alleles (1, 2) and a weak paternal allele (3). The cultured AF cells no longer show a paternal allele, but only both maternal alleles (1, 2).

diagnosis (Zadeh et al.,1987; Schwartz et al.,1989; Chitayat et al.,1995). Sometimes it poses a serious problem for genetic counselling, since we must distinguish between mosaicism, pseudomosaicism, CPM and true trisomy 9 (Hsu and Perlis, 1984; Pfeiffer et al., 1984; Appelman et al., 1991; Saura et al., 1995).

Prenatal detection of trisomy 9 mosaicism in amniotic fluid can be complicated by the absence of the trisomic cell line in different tissues and the subsequent lack of fetal anomalies in some cases (Hsu and Perlis, 1984; Merino et al., 1993; Pfeiffer et al.,1984). These authors suggested that the trisomic cells may be limited to fetal membranes. In our study, subsequent cytogenetic investigations in a compartment other than that in which the abnormality was encountered originally and an ultrasound scan were carried out to raise the significance of our prediction of the chromosomal state of the fetus.

Especially in the case of subsequent investigations, rapid detection of chromosome (9) abnormalities is particularly important. In this study, we demonstrated that a large number of interphase nuclei can be screened quickly for the presence of a

trisomic cell line in a subsequent tissue sample (uncultured amniocytes or fetal blood) within 2 days since metaphases are not necessary for FISH analysis, which eliminates the time waiting for tissue culture. Furthermore, relatively low levels of mosaic chromosome abnormalities could be detected. It was also shown that the application of FISH with a chromosome 9-specific probe has a predictive value and can aid in the counselling.

Trisomy 9 observed in CV may reflect generalized mosaicism or may indicate mosaicism confined only to the placenta. CPM has been observed at different stages of embryonic development and is associated with variable pregnancy outcomes, including spontaneous abortions, IUGR and normal birth (Appelman et al., 1991; Ledbetter et al., 1992; Saura et al., 1995). Another factor is that pregnancies with CPM are at an increased risk of fetal UPD. The incidence of fetal UPD depends on the chromosome concerned; most CPM 7 cases are probably due to somatic duplication and not to trisomic zygote rescue with a concomitant low risk of fetal UPD (Kalousek et al., 1996). However, most CPM 16 cases are due to trisomic zygote rescue and carry a 33% risk of fetal UPD (Kalousek et al., 1993). No imprinting effects of UPD 9 are known up to now (Ledbetter and Engel, 1995).

In this study, all three cases investigated showed the presence of two maternal alleles in the trisomic and in the disomic cell line compatible with a meiotic origin of the supernumerary chromosome 9 and subsequent partial trisomic zygote rescue. The absence of the paternal allele in cultured AF could be due to loss of the trisomic cell line in prolonged culturing of AF cells. Despite the rarity of chromosome 9 aneuploidy, one postnatal and one prenatal case of maternal UPD 9 have been reported before (Willat et al., 1992; Wilkinson et al., 1996). So, there seems to be a tendency for chromosome 9 aneuploidy to result in maternal UPD 9.

This study indicates that caution should be taken in handling prenatal cases with (mosaic) trisomy 9; essential steps are presented to provide maximal information to facilitate laboratory interpretations and clinical decisions.

We suggest that generalized mosaicism, pseudomosaicism or CPM concerning chromosome 9 may be differentiated from each other and result in appropriate clinical management only when the prenatal analysis is extended to include: (1) FISH studies conducted on a large number of interphase cells in dishes with and without trisomy 9 colonies, (2) FISH studies on direct cell preparations from another compartment, (3) karyotyping from another tissue and/or compartment, (4) ultrasound investigation.

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Chapter 6.2

Tetraploidy mosaicism in chorionic villi

PREVALENCE OF TETRAPLOID METAPHASES IN SEMIDIRECT AND CULTURED CHORIONIC VILLI

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Abstract

Objective: Investigation of the normal frequency of tetraploid metaphases in semidirect (STC) and cultured (LTC) chorionic villi.
Methods: Fifty metaphases in STC- and in LTC-villi slides of 100 women of advanced maternal age were screened for tetraploidy.
Results: Up to three tetraploid metaphases were encountered in 27% of the STC-villi preparations; the scores fitted a Poisson distribution. In all LTC-villi preparations tetraploid cells were seen; the scores fitted a log-Gaussian distribution.
Conclusions: On the basis of these distributions, we propose a protocol for the management of tetraploid metaphases in chorionic villi, strongly reducing the number of prenatal follow-up investigations.

Introduction

Tetraploidy in full or mosaic state is a very rare cytogenetic abnormality in liveborn infants [1]. In in-vitro cultured embryos it is a frequently observed phenomenon [2-4] and among spontaneous abortions it comprises up to 10% of the chromosomal abnormalities [5, 6]. Because of its rarity after the abortion period, only a few prenatally detected cases have been reported [7-10].

Some tetraploid cells or colonies are regularly encountered in cultured amniotic fluid cells and regarded as culture artifacts [11, 12]. Furthermore, the prevalence of tetra-ploidy and tetraploidy/diploidy mosaicism in cultured amniotic fluid cells has been well documented [12, 13]. However, a number of collaborative studies on chromosome mosaicism and pseudomosaicism in amniotic fluid cells did not mention diagnostic problems involving tetraploidy [14-17]. For chorionic villi, such experiences or basic data are not available. Therefore, we investigated retrospectively the proportion of tetraploid metaphases in trophoblast cells and cultured cells of the mesenchymal core of 100 chorionic villi samples, in order to establish the normal range for tetraploidy in chorionic villi. The purpose of this study was to provide guidelines for the differentiation of true mosaicism from placental confined mosaicism and culture artifacts.

Material and Methods

The slides of semidirect (short-term culture, STC) and cultured (long-term culture, LTC) chorionic villi of 100 pregnant women were screened for diploid and tetraploid metaphases. The indication for prenatal investigation was advanced maternal age (≥36 years) in all women. Chorionic villus sampling was performed transabdominally. Gestational age ranged from 11.0 to 13.5 weeks. At least 20 mg of villi were obtained in all cases.

STC- and LTC-villi slides were prepared according to standard techniques [18, 19]. The LTC slides were harvested in situ after 5-7 days of culturing. STC- and LTC-villi slides were investigated after Pancreatin-Trypsin-Giemsa staining. Routine cytogenetic investigation involved the karyotyping of 8 cells in STC- as well as in LTC-villi preparations. For the tetraploid investigation up to 50 metaphases in both STC- and LTC-villi slides were screened for tetraploid and diploid metaphases without discarding cells displaying low quality chromosomes or potentially incomplete metaphases.

The distributions of tetraploid cell scores in STC and LTC villi were investigated with χ^2 statistics and the Kolmogorov-Smirnov (K-S) test. Further statistics comprised calculations or the use of tables for binomial, Gaussian, and Poisson-distributions.

The outcome of all pregnancies was ascertained.

Results

Routine cytogenetic investigation revealed normal karyotypes in STC and LTC villi in 99 cases. In one instance, a non-mosaic trisomy 21 was encountered. The aim of scoring 50 metaphases was not always achieved: in STC villi, a mean of 47.5 cells (range 25-50) and in LTC villi, a mean of 48.1 cells (range 25-50) were investigated. The tetraploid cell scores are presented in figure 1. In STC-villi slides, up to three tetraploid metaphases were encountered in 27 cases (27%). The scores of tetraploid metaphases fitted a Poisson distribution (χ^2=101.26, df=99; 0.05<p<0.95) with a mean cell score of 0.31 and a variance of 0.317. The 95% (and also the 99%) area ranges from 0 to 3 cells (0- 6%). In LTC-villi slides, all 100 cases (100%) showed tetraploid metaphases. The cell scores in LTC villi fitted a log-Gaussian distribution (K-S test; 0.05<p<0.95) with a median cell score of 9 cells and a 95% area between 2 and 28 cells (4-58 %).

Ninety-six children were born without congenital malformations. One child displayed bilateral pes equinovarus, and another child a unilateral accessory auricle. The pregnancy with trisomy 21 was terminated by means of suction curettage. The trisomy 21 was confirmed; no tetraploid cells were noted in fetal fibroblasts. In another pregnancy, fetal death occurred after the chorionic villus sampling.

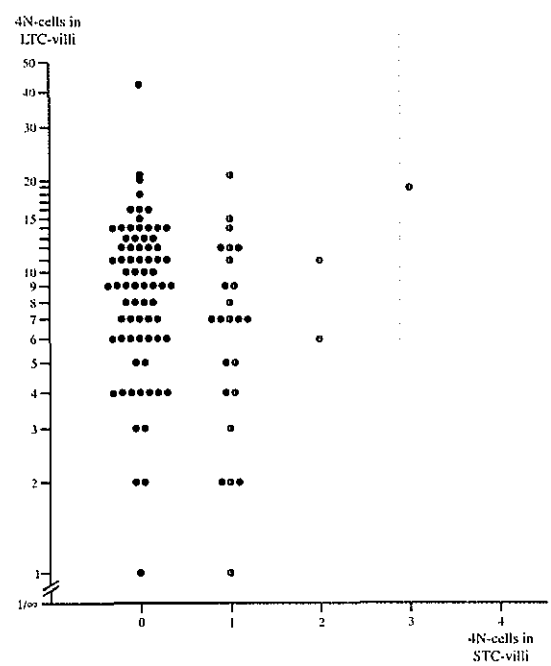

Figure 1 - Tetraploid (4N) cell scores in STC-villi (X-axis) and LTC-villi (Y-axis)

Discussion

Tetraploidy is known as a rare cytogenetic abnormality with an associated abnormal phenotype (constitutional tetraploidy), and as a frequently occurring phenomenon in cell culture (artificial tetraploidy). Moreover, tetraploid cells may be seen in STC villi under normal conditions representing confined placental mosaicism[20-28]. In our study, the tetraploidy cell scores in STC and LTC villi fitted a Poisson and log-Gaussian distribution, respectively. This remarkable difference in distributions finds its orgin in the cell culture.

Constitutional tetraploidy arises in the first four post-zygotic cell divisions by cytokinesis failure, endo-reduplication, or nuclear fusion in binucleated blastomeres [2, 3]. Once formed, tetraploid cells can undergo correction to normal diploid blastomeres again [4]. After the 8-cell stage, embryonic cells are distributed among the compartments of the trophoblast and the inner cell mass (ICM). From the latter compartment, the future extraembryonic mesoderm, which is investigated in LTC villi, and the fetus proper will arise [29]. Tetraploid cells are almost exclusively allocated to the trophoblast compartment in the human embryo [2, 30]. In the case of constitutional tetraploidy in a vital pregnancy, the lowest level of tetraploidy mosaicism in STC and LTC villi is theoretically 33.3% assuming that a skewed distribution of tetraploid cells towards the ICM compartment is not compatible with embryo development [31]. Furthermore, we assume that a 50% reduction occurs in the number of daughter cells of blastomeres involved in tetraploid formation compared to normal diploid blastomeres. A cell score of 9 tetraploid cells per 47.5 investigated cells (19%) in STC villi represents the lower limit of the 95% area of a 33.3% mosaicism and finds itself far outside the 99% normal area of 0-3 cells. When a cell score of at least 9 cells in STC villi is accompanied by a tetraploidy cell score in the LTC villi of at least 11 per 48.1 analyzed cells (23%), constitutional tetraploidy in the fetus proper has to be excluded or demonstrated.

Table 1 - Management of the finding of tetraploid (4N) cells in chorionic villi

	Condition	Action
1	Observing ≥2 4N-cells in search for 8 analyzable cells in STC-villi	Screening 50 metaphases in STC-villi for 4N-cells
2a	≤8 4N-cells/50cells in STC-villi	Routine investigation of 8 analysable cells in LTC-villi
2b	≥9 4N-cells/50 cells in STC-villi	Screening 50 metaphases in LTC-villi for 4N-cells
3a	≥9 4N-cells/50 cells in STC-villi ≤10 4N-cells/50 cells in LTC-villi	Normal cytogenetic results in chorionic villi
3b	≥9 4N-cells/50 cells in STC-villi ≥11 4N-cells/50 cells in LTC-villi	Amniocentesis; FISH on uncultured AF-cells

AF= amniotic fluid; FISH=fluorescent in situ hybridization

This minimum LTC-villi cell score of 11 cells is composed of 9 cells (19%) representing again the lower 2.5% limit of a theoretical 33.3% mosaicism of constitutional tetraploidy and 2 cells (4%) representing the lower 2.5% limit of the 95% area of artificial tetraploidy.

With these figures we developed a three-stage protocol for the management of tetraploid metaphases in chorionic villi (table 1). For follow-up investigation in a repeat sample we would advise amniocentesis with fluorescent in situ hybridization on uncultured amniotic fluid cells; only in this way can the problem of culture-induced tetraploidy be overcome.

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Chapter 6.3

Isochromosome 18p and 18q formation

PRENATAL DETECTION OF TRISOMY 18 CAUSED BY ISOCHROMOSOME 18p AND 18q FORMATION

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Abstract

We report on the prenatal detection and further genetic studies in a case of trisomy 18 caused by isochromosome 18p [i(18p)] and 18q [i(18q)] formation. The diagnosis was made by standard cytogenetic techniques in amniotic fluid cells and confirmed by fluorescence in situ hybridization. The formation of the isochromosomes cannot be explained by a single model; centromere misdivision and meiosis II nondisjunction without recombination or mitotic misdivision are the most likely mechanisms of formation as indicated by DNA analysis.

Introduction

Several cases of prenatally detected isochromosome 18 have been reported: (mosaic) i(18q) [Chen et al., 1998; Froster-Iskenius et al.,1984; Levy-Mozziconacci et al.,1996; Qumsiyen et al.,1995; Speed,1986; Sutton and Ridler,1986; Wurster-Hill et al.,1991] and (mosaic) i(18p) [Darnaude et al.,1996; Göcke et al.,1986; Pinto et al.,1998; Yu et al.,1993].

Two cases of trisomy-18 syndrome due to double isochromosome formation have been reported [Larson et al.,1972; Müller et al.,1972]. To our knowledge, only one prenatally detected case with isochromosomes for both p and q arms [i(18q) + i psu dic(18p)] has been published [Romain et al.,1992]. We describe an almost identical case: the prenatal detection of a 47,XX,-18, + i(18p) + i(18q) karyotype in amniotic fluid cells investigated with conventional cytogenetic techniques, followed by fluorescence in situ hybridization (FISH) for the definite identification of the isochromosomes.

DNA analyses to determine the mechanism of formation of the isochromosomes 18p and 18q and the parental origin were carried out.

Clinical Report

A 35-year-old pregnant woman (G3, P1, Ab1) was referred for prenatal diagnosis because of a positive result of 2nd trimester maternal serum screening for Down syndrome and neural tube defects [Beekhuis et al.,1992]. At 15.5 weeks of gestation, the maternal serum alpha fetoprotein (MSAFP) and human chorionic gonadotropin (MShCG) levels were at 0.88 and 2.10 multiples of the median (MOM), respectively, resulting in a risk for fetal Down syndrome of 1:200. The family history of the woman and her 35-year-old husband was unremarkable. Amniocentesis was performed at 16.5 weeks of gestation. There were no fetal abnormalities noted by ultrasound investigation at the time of screening and later at amniocentesis. After the finding of one normal chromosome 18 accompanied by two isochromosomes [i(18p) and i(18q)], likely causing trisomy-18 syndrome, a repeat detailed fetal ultrasound at 17.5 weeks again showed no abnormalities. The parents opted for termination of pregnancy.

Labour was induced and a stillborn female fetus of 215 g was delivered (mean for 17.5 weeks is 200 g)[Chambers et al., 1993]. On physical examination, some external abnormalities were noted like hypoplastic maxilla, micrognathia, and a prominent nose giving a bird-like appearance (Fig. 1). The placenta was incomplete and the remnants had been removed by curettage; so, no data on placental weight are available.

The parents consented to confirmatory studies on a skin biopsy and placental biopsy, but not to an autopsy of the fetus.

Figure 1 - Frontal view of the fetus at 17.5 weeks, showing the prominent nose and the hypoplasia of the maxilla and the micrognathia giving a bird-like appearance

Genetic Studies

Amniotic fluid cells were cultured using standard procedures. Chromosomes were analyzed by Trypsin-Giemsa banding. The karyotype was 47,XX,-18, + i(18p) + i(18q) in all 16 investigated clones (Fig. 2a). In fetal fibroblasts and chorionic villi (both short-term and long-term culture) this karyotype was confirmed in all investigated cells. Karyotypes of the parents were normal 46,XY and 46,XX, respectively.

FISH was performed on unstained slides of cultured amniocytes with a whole chromosome 18 paint (WCP 18)(Cambio Ltd., Cambridge, UK), a chromosome 18 centromeric probe (18cen), L1.84 [Devilee et al., 1986] and the telomeric probes 18pter, 52M11 and 18qter, 2050a6 [National Institutes of Health and Institute of Molecular Collaboration, 1996]. Hybridization with WCP 18 was done according to the procedure recommended by the manufacturer. A three-color FISH with a combination of biotin- and digoxigenin-labeled 18cen probe (yellow), biotin-labeled 18qter probe (red) and digoxigenin-labeled 18pter probe (green) was done according to standard protocols. Slides were examined under a Leica aristoplan fluorescence microscope and images were captured by the Genetiscan Power Gene System (Perceptive Scientific Instruments Ltd., Chester, U.K.). Hybridization with WCP 18 resulted in a fluorescent staining of the normal chromosome 18 and both isochromosomes (Fig. 2b). Positive hybridization signals were seen with L1.84 and 52M11 and with L1.84 and 2050a6, confirming the isochromosomes to be i(18p) and i(18q) (Fig.2c), respectively. Additionally to the 16 karyotyped clones, interphase nuclei were screened for the signal distribution with L1.84 in 17 clones without analyzable metaphases; in all clones three spots were counted, proving the presence of the abnormal chromosomal constitution in 33 clones.

DNA was extracted from cultured amniotic fluid cells and blood cells of both parents using standard methodology. A total of 21 microsatellite loci were analyzed using the polymerase chain reaction (PCR) to determine the mechanism of formation

and the parental origin of the isochromosomes. PCR analysis demonstrated only one maternal and one paternal allele of all investigated markers (Table I). The absence of detectable recombination precluded the certain establishment of the parent of origin. However, the intensity of bands suggested that the isochromosomes were of maternal origin.

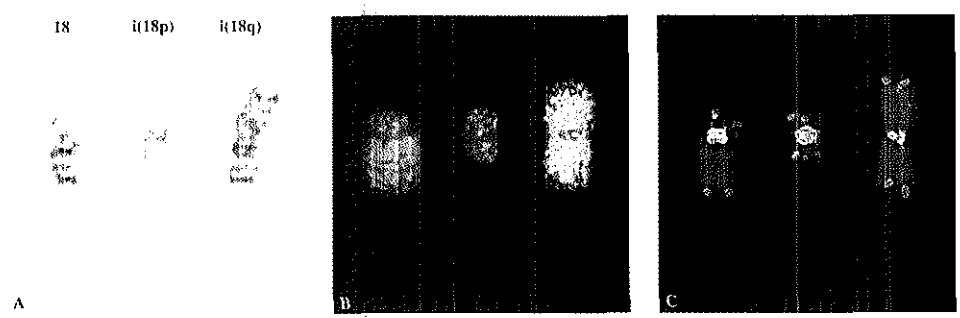

Figure 2 - Partial karyotype of cultured amniotic fluid cells; **(A)** Trypsin Giemsa staining. FISH signals on normal chromosome 18, i(18p) and i(18q) with **(B)** whole chromosome paint 18 and **(C)** 18 centromere probe L1.84 (yellow), 18pter probe 52M11 (green), 18qter 2050a6 (red).

Table I - DNA analysis to determine the mechanism of formation and the parental origin of the isochromosomes i(18p)and i(18q). The loci are ordered according to their chromosomal location

Locus	Location	Father	Mother	Fetus
D18S59	p11.32-pter	2,3	1,3	1,3
D18S476	p11.32	1,2	3,3	2,3
D18S1154	p11.32	3,4	1,2	2,4
D18S452	p11.31	2,3	1,2	2,3
D18S52	p11.22	1,2	1,3	1,3
D18S1153	p11.22	1,2	2,2	1,2
D18S53	p11.21-p11.22	2,3	1,4	2,4
D18S71	p11.21	1,2	1,2	1,2
D18S40	p11.21	3,4	1,2	1,3
D18S57	q12.2	1,4	2,3	3,4
D18S1157	q12.3	3,4	1,2	2,4
D18S42	q21	1,2	2,3	2,3
D18S64	q21.32	2,2	1,1	1,2
D18S68	q22.1	1,2	3,4	2,4
D18S483	q22.1	1,1	2,2	1,2
D18S61	q22.3	2,2	1,2	2,2
D18S488	q22.3	2,4	1,3	3,4
D18S43	q22-q23	2,3	1,2	1,2
D18S1161	q23	2,4	1,3	1,4
MBP	q22-qter	2,3	1,1	1,3
D18S70	q23	1,2	3,4	2,3

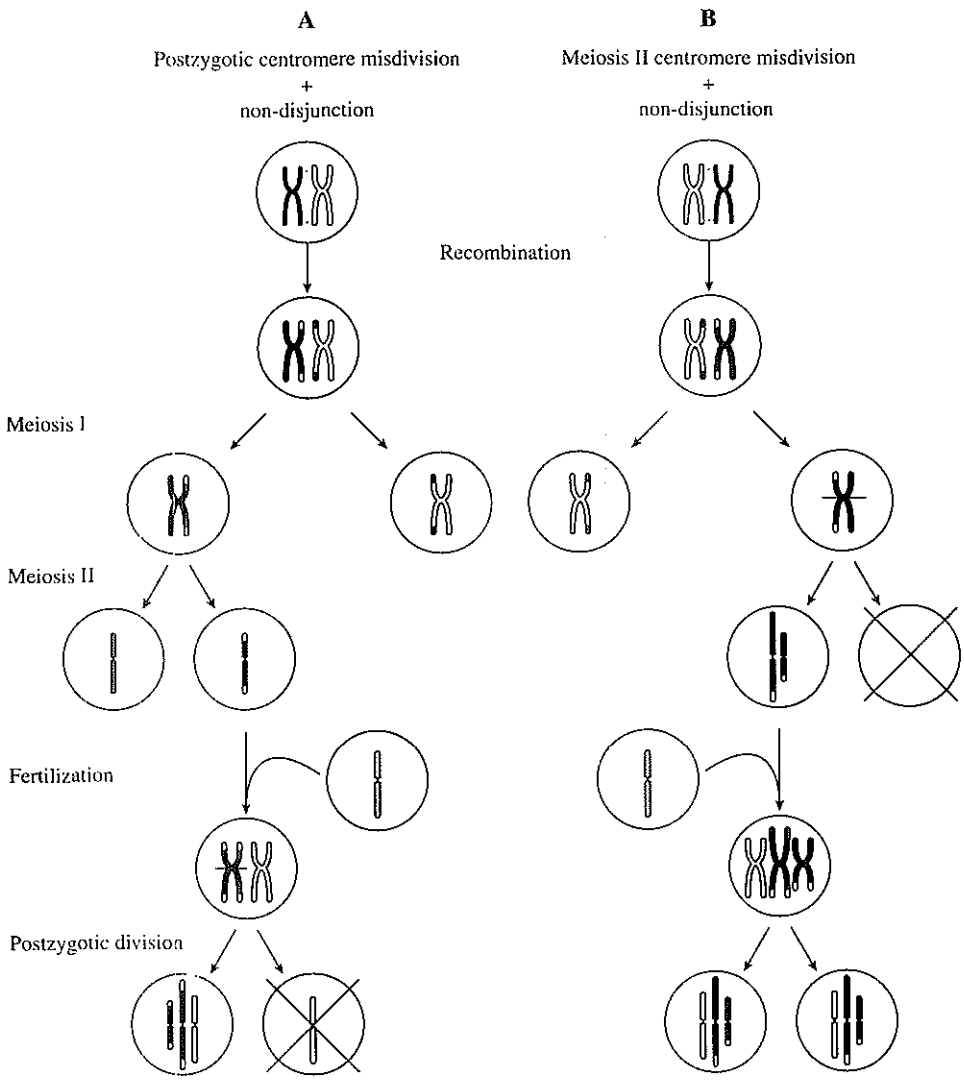

Figure 3 - Schematic representation of possible mechanisms of isochromosome 18p and 18q formation; **(A)** Recombination before Meiosis I followed by postzygotic centromere misdivision and nondisjunction resulting in isochromosomes with complete isodisomy; **(B)** Recombination before Meiosis I followed by centromere misdivision and nondisjunction in Meiosis II resulting in isochromosomes with partial heterodisomy.

Discussion

The isochromosomes 18p and 18q in this prenatal case were present in addition to one normal chromosome 18 in all cells, resulting in trisomy 18. Although the phenotype may be quite variable, many typical signs such as growth retardation, low-set ears, clenched fists with overlapping fingers, and rocker bottom feet were absent in the present case. Mosaicism as explanation was ruled out by the finding of trisomy 18 in all 33 present clones.

The indication for prenatal diagnosis was a screen-positive result for fetal Down syndrome because of a rather elevated MShCG level. In general, trisomy 18 is associated with very low MShCG levels [Aitken et al., 1996; Lambert-Messerlian et al., 1998]. The high concentration of MShCG in this case is difficult to explain and possibly related to the rare chromosomal composition of the trisomy 18, the unusual fetal phenotype and the absence of growth retardation.

Several mechanisms of isochromosome formation have been postulated. The two most- common mechanisms are misdivision of the centromere, resulting in mono-centric products [Darlington 1939, 1940] and the U-type reunion between sister chromatids, resulting in dicentric or mono-centric products [de la Chapelle, 1982]. Although isochromosomes are defined as cytogenetically identical copies of the same chromosome arm, recombination could be expected to result in heterozygosity for markers especially in the telomeric region, in case of a meiotic origin [Bugge et al., 1996; Eggermann et al., 1997; Kotzot et al., 1996]. In our case recombination was not detected. Molecular genetic analysis utilizing polymorphic markers which map to both the short and the long arm of chromosome 18 failed to demonstrate the presence of three distinct alleles in the fetus at any locus analyzed. Although a meiosis II centromere misdivision followed by anondisjunctional error without previous meiosis I recombination cannot be ruled out, the most likely hypothesis regarding the formation of these isochromosomes, appears to be a postzygotic centromere misdivision followed by a nondisjunctional error (Fig.3) since no recombination was observed at any of these loci.

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Chapter 6.4

Non-mosaic discordance between STC-villi and LTC-villi

A CASE OF 45,X/46,XY MOSAICISM WITH NON-MOSAIC DISCORDANCE BETWEEN SHORT-TERM VILLI (45,X) AND CULTURED VILLI (46,XY)

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Abstract

We report on a prenatally detected case of discordant non-mosaic karyotypes following chorionic villus sampling. A 45,X karyotype was found in cytotrophoblast cells and a 46,XY karyotype in mesenchymal core cells. A subsequent amniocentesis showed a true 45,X/46,XY mosaicism. Confirmatory studies, including fluorescence in situ hybridization (FISH) in various fetal and placental tissues as well as in the original villi preparations changed the presumed condition of generalized mosaicism with culture confined normality to that of generalized mosaicism with absolute concordance.

This case underscores the importance of the investigation of both short-term and cultured villi preparations, the implementation of prenatal FISH studies, and the need for thorough follow-up investigation in cases of discrepant results.

Introduction

Several reports have been published on cases in which the karyotypes of short-term cultured (STC-villi) and long-term cultured villi (LTC-villi) did not correspond to each other or to that of the fetus [Ledbetter et al., 1992; Kalousek et al., 1994; Leschot et al., 1996; Hahnemann and Vejerslev, 1997a, 1997b]. However, the prenatal cytogenetic observation of an abnormal karyotype in STC-villi, a normal karyotype in LTC-villi, and an abnormal karyotype again in the fetal compartment is extremely rare [Pittalis et al., 1994; Kennerknecht et al., 1998]. This constitution is known as generalized mosaicism with discordant culture (confined culture normality)(GMDC)[Pittalis et al., 1994]. Theoretically, GMDC can occur as a result of the distribution of normal and abnormal cells among the compartments of the trophoblast, extra embryonic mesoderm (EEM) and fetus [Los et al., 1998].

We present a case of 45,X/46,XY mosaicism, initially thought to represent an example of GMDC. Routine cytogenetic analysis showed a 45,X karyotype in STC-villi and a 46,XY karyotype in LTC- villi. Subsequent amniocentesis disclosed a 45,X/46,XY mosaicism which was confirmed in various fetal and placental tissues. Extensive chromosome and fluorescence in situ hybridization (FISH) investigations were carried out not only to gain insight into this cytogenetic inconsistency but also to find an explanation for this observation based on the embryogenic models presenting combinations of different karyotypes in the various fetal compartments [Crane and Cheung, 1988; Bianchi et al., 1993; Wolstenholme, 1996; Los et al., 1998].

Case report

A 35-year-old woman, gravida 4, para 3, underwent transabdominal chorionic villus sampling at 12 weeks of gestation because of maternal anxiety. Medical and family histories were unremarkable.

Cytogenetic analysis of cytotrophoblast cells (STC-villi) and of mesenchymal core cells (LTC-villi) were performed according to standard techniques [Gibas et al., 1987; Smidt-Jensen et al., 1989].

STC-villi showed a 45,X karyotype in all 31 investigated cells. The karyotype of LTC-villi was 46,XY in all 24 investigated cells. An amniocentesis was offered and undertaken at 16 weeks of gestation to resolve this discrepancy. Ultrasound examination at the time of amniocentesis demonstrated a male fetus without structural abnormalities.

FISH on uncultured amniocytes was carried out as described [Van Opstal et al., 1993] with a combination of a centromere probe for chromosome X (pBamX5)[Willard et al., 1983] and a Y heterochromatine probe (RPN1305X)[Lau, 1985]. FISH on uncultured amniocytes demonstrated a X/XY mosaicism which was subsequently confirmed in cultured amniocytes. The diagnosis of true mosaicism for sex chromosomal aneuploidy was made. The woman was counseled that there was a

small risk of abnormal external genitalia but a significant higher risk of abnormal internal genitalia (ovatestes); she requested termination of pregnancy.

The incidence of GMDC is extremely rare [Pittalis et al., 1994]. Subsequent FISH investigations on STC- and LTC-villi were carried out to gain insight into this mosaicism. Interphase FISH showed a low X/XY mosaicism in LTC-villi and the prenatal diagnosis, therefore, changed from the presumed condition of GMDC to that of generalized mosaicism with relative concordance (GMRC). GMRC is characterized by a homogeneous abnormal karyotype in at least one of the three investigated compartments of STC-villi, LTC-villi and amniocytes, and a mosaic karyotype in the other compartment(s)[Pittalis et al., 1994]. A summary of chromosome and FISH results is given in Table I.

Table I - Summary of prenatal chromosome and FISH analysis

		Percent of X and XY signals in metaphases			Percent of X and XY signals in interphases		
Tissue	Karyotype [*N*]	*N*	X	XY	*N*	X	XY
STC-villi	45,X[31]	31	100	-	200	100	-
LTC-villi	46,XY[24]	24	-	100	200	20	80
Uncultured amniocytes	-	-	-	-	200	16	84
Cultured amniocytes	45,X[3]/46,XY[20]	50	100	88	200	23	77

N=number of metaphases and interphase nuclei

Table II - Summary of confirmatory chromosome and FISH analysis

		Percent of X and XY signals in metaphases			Percent of X and XY signals in interphases		
Tissue	Karyotype [N]	N	X	XY	N	X	XY
Skin	45,X[1]/46,XY[49]	50	8	92	200	22	78
Left gonad	45,X[4]/46,XY[46]	50	14	86	190	10	90
Right gonad	45,X[2]/46,XY[47]	50	18	82	200	25	75
Left kidney	45,X[5]/46,XY[45]	50	8	92	200	24	76
Left lung	45,X[2]/46,XY[48]	50	8	92	200	5[a]	95
Cultured amnion	46,XY[50]	50	4	96	200	3	97
Cultured chorion	46,XY[25]	10	-	100	200	1	99
STC-villi 1	-	-	-	-	100	97	3
STC-villi 2	-	-	-	-	50	34	76
STC-villi 3	-	-	-	-	100	77	33
STC-villi 4	-	-	-	-	100	17	83
LTC-villi 1	-	10	-	100	200	-	100
LTC-villi 2	-	50	12	88	200	10	90
LTC-villi 3	-	17	-	100	200	11	89
LTC-villi 4	-	10	-	100	200	2	98

N = number of metaphases and interphase nuclei; a = 1-5% with only X signal is within the normal range.

Postmortem examination showed a male fetus with retrognathia, a broad nasal bridge and a broad philtrum, but no other abnormalities. Normal male genitalia were present without any sign of female differentiation. Biopsies of chorionic villi (four sites), amniotic- and chorionic membranes, and fetal biopsies (skin, lung, kidney, left and right gonad) were sampled for both cytogenetic and FISH analysis. Table II summarizes the confirmatory chromosome and FISH analysis. All fetal biopsies showed X/XY mosaicism on both metaphase and interphase level. Amniotic- and chorionic membranes showed a XY cell line. FISH on STC-villi showed a surprisingly high level of XY signals, whereas the LTC-villi displayed a low level of X signals only, in two of the four sites. So finally, the presumed condition of GMRC turned to that of generalized mosaicism with absolute concordance (GMAC), mosaic abnormal karyotypes in all three investigated compartments [Pittalis et al., 1994].

Discussion

An abnormal cell division in early embryonic development may cause chromosomal mosaicism that can result in discrepancies between chorionic villi and the fetus proper.

The present case demonstrated a non-mosaic abnormal karyotype in STC-villi and a normal karyotype in LTC-villi that could fit into a confined placental mosaicism (CPM) type I in which the abnormal cell line is confined to the cytotrophoblast or into a GMDC in which the normal cell line is confined to the compartment of cultured villi. The incidence of the latter is probably very low, making CPM type I more likely [Pittalis et al., 1994]. There are several studies that make mention of the restricted reliability of cytogenetic diagnosis of mosaic and even non-mosaic monosomy X in CVS [Pittalis et al.,1994; Hahneman and Vejerslev, 1997b; Smith et al., 1999]. A definite prenatal diagnosis should be obtained through subsequent amniocentesis.

However, in the present case, FISH on uncultured amniotic fluid cells showed a X/XY mosaicism that was confirmed by the chromosome analysis of the cultured cells. These findings fitted into one of the two models suggested, GMDC, an extremely rare finding. FISH provided the opportunity to investigate a larger number of cells than using conventional cytogenetic methods. Extensive FISH investigations on all biopsies described in Table II as well as on the prenatal STC- and LTC-villi (Table I) showed a (low) mosaicism in all compartments which demonstrated the GMDC to be in fact the cytogenetic constitution of GMAC.

The observation of placental site variation of abnormal cells is not uncommon and indicates that the findings in STC- and LTC-villi are not always representative for the fetus [Miny et al., 1991; Schuring-Blom et al., 1993; Henderson et al., 1996].

To understand the cytogenetic discrepancies between STC-villi, LTC-villi and amniocytes, it is necessary to implement the knowledge of early embryonic development and the origins of these different tissues [Wolstenholme, 1996]. An accurate examination of all three compartments i.e. the cytotrophoblast, mesodermal

core, and fetus was needed to come to a clearer explanation for the chromosomal constitution of the present case (Table I and II).

According to the embryogenic model given by Crane and Cheung [1988] explaining cytogenetic discrepancies in different compartments we propose that the karyotype of the zygote in this particular case was 46,XY. To explain the combination of karyotypes and the levels of abnormal cells in different tissues we propose two theoretical modes of origin. The first mode comprises one event: anaphase lagging in the first cleavage resulting in one 45,X and one 46,XY cell (Fig. 1A). In the second mode two independent anaphase laggings are proposed, one in the first and one in the second cleavage leading to three 45,X cells and one 46,XY cell (Fig. 1B). Although the second mode is more complicated and obviously rare it seems the most likely mechanism in view of the rarity of GMDC reports in the literature. We propose most of the 45,X cells to be allocated to the cytotrophoblast and most of the 46,XY cells to the inner cell mass (ICM) due to a selective disadvantage of 45,X cells predominating in the ICM and subsequently in the fetus.

This present case not only demonstrates the need for extensive follow-up investigation after the finding of non-mosaic discordant results in STC- and LTC-villi but also demonstrates the crucial role of FISH analysis to get more insight into discrepancies in the various fetal compartments.

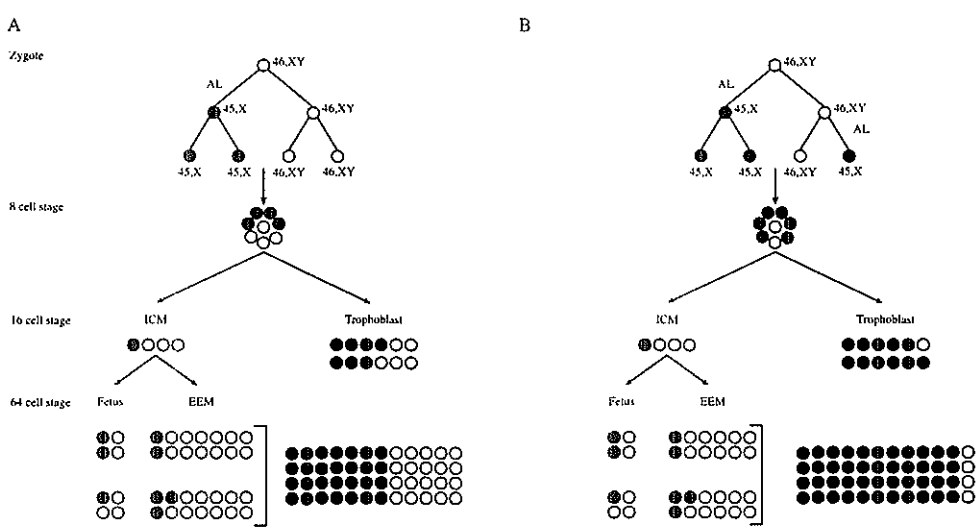

Figure 1 - Mode of origin explaining cytogenetic inconsistencies. **A:** Anaphase lagging (AL) in 1st cell cleavage and theoretical distribution of abnormal and normal cells. **B:** Anaphase lagging in 1st and 2nd cell cleavage and theoretical distribution of abnormal and normal cells. ICM = Inner Cell Mass; EEM = Extra Embryonic Mesoderm.

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Chapter 7

General discussion and future prospects

7.1 General discussion

Prenatal cytogenetic diagnosis is performed on amniotic fluid (AF) cells or chorionic villi and occasionally on fetal lymphocytes obtained by cordocentesis. The main disadvantages of cytogenetic investigation on cultured AF-cells are the advanced gestational age (> 15 weeks) at sampling and the long reporting time of laboratory results (2-3 weeks). The procedure related risks of pregnancy loss, intra-uterine fetal death, and induction of congenital abnormalities have proven to be low (MRC Working Party, 1978; Tabor et al.,1986).
After the introduction of chorionic villus sampling in 1983 (Simoni et al., 1983) it was believed that this new technique with its favourable early, first-trimester sampling, and short reporting time of laboratory results would eventually replace amniocentesis. However, due to a supposed higher abortion risk in comparison to amniocentesis (Rhoads et al., 1989; Smidt-Jensen et al., 1992), and the ongoing argument of the induction of vascular disruptive syndromes (Froster and Jackson, 1996; Los et al., 1996; Firth, 1997) this has not been the case.
Furthermore, doubt has arisen about the accuracy and reliability of cytogenetic laboratory results of chorionic villi (Hahnemann and Vejerslev, 1997a). Hence, amniocentesis andsubsequent cytogenetic investigation of cultured AF-cells has remained the main mode of prenatal cytogenetic diagnosis, and has become the "gold standard" (Stranc et al., 1997). Because of the obvious clinical advantages of first trimester diagnosis, a thorough evaluation of the diagnostic performance of cytogenetic investigation in chorionic villi was made in this thesis and compared to that of AF-cells, in order to come to fair and evidence- based recommendations for the use of amniocentesis and chorionic villus sampling.

7.1.1 Cytogenetic investigation in STC-villi

The first aim of the experimental work comprised in this thesis was to examine the reliability of prenatal diagnosis in chorionic villi with the investigation of STC-villi alone. The most important questions to be answered were:
- What is the predictive value of the cytogenetic result in STC-villi.
- Can recommendations be made for the prenatal diagnosis using chorionic villi.

Predictive value
The predictive value of abnormal cytogenetic results of STC-villi turned out to depend on the indication and the type of chromosome aberration found.
The best results were achieved in women with the highest cytogenetic risks: maternal age ≥40 years, carriership of structural rearrangements, and fetal abnormalities established by ultrasound.
Apart from normal results, the predictive value was found to be high in case of trisomy 21, triploidy, expected structural rearrangements and in case of ultrasound-abnormalities in trisomy 13, 18 and 45,X. Furthermore, it was shown that the predictive

value for any category of numerical chromosomal aberration is limited if the aberration is mosaic.
The confirmation rate of mosaic cases is dependent on the level of mosaicism; a high level of mosaicism has a higher predictive value than a low level of mosaicism.

Recommendations
We advise chorionic villus sampling to those women with singleton pregnancies who have a cytogenetic risk of at least 3%, especially when only STC-villi preparations are investigated. Furthermore, we advice chorionic villus sampling to those women with multiple gestations.
Cytogenetics on chorionic villi should be carried out on STC- and LTC-villi preparations simultaneously in order to reduce the number of uncertain results requiring follow-up investigations. In various retrospective studies, karyotyping of STC- as well as LTC-villi preparations has already been recommended (ACC, 1994; Pittalis et al., 1994; Hahneman and Vejerslev, 1997a). Furthermore, the analysis of LTC-villi should not only reduce the risk of false-positive results and eliminate the theoretical risk of false-negative results but also improve the quality of the fetal karyotype.

7.1.2 *Cytogenetic investigation in STC- and LTC-villi*

After the recommendation that cytogenetics should be carried out on STC-villi and LTC-villi simultaneously, the subsequent aim of the work described in this thesis was the prospective evaluation of the combined cytogenetic results of STC- and LTC- villi.
During the evaluation of the combined use of STC- and LTC-villi we focussed on the following questions:
- Is there an improvement of the chromosome quality in comparison to the analysis of STC-villi alone.
- Are the simultaneous investigation of STC- and LTC-villi more representative for the fetus proper than the analysis of STC-villi alone.
- Is the predicted reduction in the rate of follow-up investigations realised.
- Can practical laboratory guidelines be put forward.

Quality of chromosome preparations
The improvement of karyotype quality is evidently shown in chapter 1, 3 and 5. Although analysing STC-villi alone did not lead to any overlooked chromosomal aberration in the past, it is our goal with the analysis both STC- and LTC-villi to optimize the chromosome quality resulting in a quality equal to that of amniotic fluid cells and, at the same time, to meet the required 400 band level for prenatal cytogenetic diagnosis.

Reliability of cytogenetic results
Large series and various collaborative studies have shown that the combined use of STC- and LTC-villi minimizes diagnostic errors and, therefore, chorionic villi are acceptable for early prenatal diagnosis (Breed et al., 1990; Hahneman and Vejerslev,

1997a, 1997b; Ledbetter et al., 1992; ACC, 1994; Van den Berg et al., 2000a). It is known that the discrepancies between the chromosomal constitution of the fetus and chorionic villi are the consequence of the arising of mosaicism in the early embryo and the subsequent distribution of normal and abnormal cells among the various compartments (Wolstenholme et al., 1996; Los et al., 1998). However, the combined use of STC- and LTC-villi allows the recognition of all possible cytogenetic combinations in chorionic villi and the fetus proper. This results in a higher predictive value of the cytogenetic result of STC- and LTC-villi in comparison to the predictive value of STC-villi alone. This is demonstrated in the proportion of certain abnormal results which has greatly improved from 50% with the analysis of STC-villi alone to approximately 75% with the combined analysis of STC- and LTC-villi.

Follow-up investigations
It is to be expected that the combined analysis of both villi compartments results in an actual and significant reduction for the need of follow-up investigations in comparison with the diagnosis of the STC-villi compartment alone. Follow-up investigations were required among 2.1% of the women investigated in chapter 2, which corresponds to another study (Leschot et al., 1996). After the introduction of the routine analysis of both STC- and LTC-villi and the implementation of all guidelines for the handling of uncertain results, follow-up investigation requiring a second invasive procedure declined significantly by more than one-third from 2.1% (1993-1996) to 1.6% (1997-1999), and finally to 1.2% in 2000.

Practical laboratory guidelines
In cases with normal karyotypes, 16 cells are analysed routinely, where in all other cases a work-up protocol is needed. In view of the literature and our own results, work-up protocols were and still are developed in our laboratory for all kind of situations of analysing STC-villi alone or both villi compartments. The work-up protocols, demonstrated in chapter 1, turned out to be very useful and efficient for the differentiation between generalized mosaicism and CPM.

7.1.3 Prenatal diagnosis in multiple gestations

A further aim was the evaluation of a particular group of patients; the women with multiple gestations which present particular problems in counselling, sampling and management.
The study question was:
- What recommendations could be made in this group of patients?

Recommendations towards multiple gestations
Compared with amniocentesis there is a somewhat higher risk of uncertain results and a greater risk of mixed sampling leading to cross-contamination in chorionic villus sampling. However, the simultaneous cytogenetic investigation of both STC- and LTC-villi will reduce the chance of uncertain results. The identification of potential cross-

contamination in cases of biochemical and/or DNA indications by multiple marker DNA analysis will eliminate the chance of misdiagnosis.
Together with the benefit of an earlier and safer selective reduction we consider chorionic villus sampling the method of choice for prenatal diagnosis in multiple gestations. This view was shared during the 10th International Conference on Prenatal Diagnosis and Therapy (Barcelona, Spain, June 2000) where Wapner stated that chorionic villus sampling is the mode of sampling of first choice in twin pregnancies.

7.1.4 Recurrent and/or specific problems in prenatal cytogenetics

In prenatal cytogenetic diagnosis recurrent and/or specific cytogenetic problems occur. In chapter 6 of this thesis some of these problems are described and suggestions about solutions are made.

Tetraploidy mosaicism in chorionic villi
A frequent recurrent phenomenon in cytogenetic diagnosis is the presence of a tetraploidy mosaicism. We developed a work-up protocol for the management of tetraploid cells in chorionic villi based on theoretical experiments and distributions of tetraploid and diploid cells in the various compartments. This protocol turned out to be a very good tool for handling this phenomenon and also resulted in a decrease of follow-up investigations from 0.7% to almost 0% (Noomen et al., 2001).

Non-mosaic discordance between STC-villi and LTC-villi
Theoretically, non-mosaic abnormal STC-villi with normal LTC-villi can result in a CPM type I or a GMDC (table 5, chapter 1). GMDC has not yet been reported with certainty in several large studies in contrast to CPM type I (Hahneman and Vejerslev 1997b; Pittalis et al. 1994; ACC 1994; Van den Berg et.al., 2000b; Ledbetter et al. 1992). A presumed case of GMDC concerning a 45,X/46,XY mosaicism turned out to be a case of GMAC thanks to follow-up and confirmatory investigations. We conclude that in cases of sex-chromosomal discordancies follow-up investigations are indicated because the experiences with those cases are limited (Smith et al., 1999, Van den Berg et al., 2000a). In all other cases (autosomal aneuploidies) one may consider follow-up investigations in case of a "XX" karyotype to rule out maternal cell contamination in the LTC-villi.

Trisomy 9
A specific problem in prenatal diagnosis is the presence of a trisomy 9 (mosaicism) in chorionic villi and AF-cells, since in some cases the abnormality could not be confirmed. The latter poses a serious problem for genetic counselling. Subsequent cytogenetic investigations in other compartments are essential for a correct prediction of the chromosomal state of the fetus. FISH turned out to be a rapid and accurate method for verification of trisomic cell lines and may provide additional information for the prenatal cytogenetic results. Comparison of the level of mosaicism in uncultured and cultured AF-cells demonstrated the rapid disappearance of the abnormal cell-line by overgrowth of the normal cells in cell culture.

Isochromosome 18p and 18q formation
Although, the diagnosis of trisomy 18 caused by isochromosomes 18p and 18q poses no problem for genetic counselling, DNA analysis was carried out to determine the mechanism of formation of the isochromosomes and the parental origin. Gaining more insight in the mechanism of formation of the isochromosomes has helped in similar cases, especially when the isochromosomes were present in combination with a normal cell line.

7.1.5 Chorionic villus sampling or amniocentesis?

Our final aim was to investigate whether a difference in the diagnostic performance of cytogenetic investigation in chorionic villi and AF-cells in favour of the last justifies the fact that chorionic villus sampling has not reached the popularity of amniocentesis. These diagnostic performances have been the subject of chapter 5. The data are summarised in Table 1. Additionally, data on the reporting time of laboratory results, the potential signalling of uniparental disomy (UPD), and on some sampling related factors are presented in Table 1 as well.
Together these data reflect the total performance in the years 1993-1999, and give the possibility to weigh the pros and cons of chorionic villus sampling and amniocentesis.

Table 1 - The total performance (1993-1999): Chorionic Villi versus Amniotic Fluid

Factor/Condition	STC-villi only 1993 - 1996	STC- and LTC-villi 1997 - 1999	Amniotic Fluid 1993 - 1999	Statistics
False positive (discrepant) diagnosis	2/3499	0/1829	0/11.753	NS
False negative (wrong) diagnosis	0/3499	0/1829	2/11.753	NS
Laboratory success rate	99.5%	99.5%	99.6%	NS
Chromosome quality (number of bands, mean ± SD)	311 ± 34.6	364 ± 32.6	373 ± 38.1	$p = 0.001$
Follow-up sampling rate	2.2%	1.6%	0.3%	$p < 0.001$
Reporting time (mean ± SD)	5.6 ±2.8	8.3 ±1.8	14.5 ±2.2	$p < 0.001$[1]
Potential UPD signalling	+	+	-	-
Desirability in multiple gestations	+	++	-	-
Procedure related risks	26/2660[2]	11/1232[2]	66/9696[2]	NS
Gestational age at sampling	10.5 - 13 wks	10.5 - 13 wks	15.5 - 17 wks	-

1 = ANOVA, $F = 20.435{,}5$, $df_1 = 2$, $df_2 = 15.681$; $p < 0.001$. 2 = singleton pregnancies, US-abnormalities excluded; amniocentesis or chorionic villus sampling in Dijkzigt Hospital, Rotterdam (unpublished data from H. Brandenburg and F.J. Los).
The shaded squares indicate the level of performance where white to dark grey represents good to poorer performance.

It has been shown in chapter 5 that the follow-up sampling rate depends on the representativity of the cytogenetic results in the prenatal sample for the actual karyotype of the fetus. Mosaicisms apear to play a major part in this rate and again in relation to the distribution of normal and abnormal cells among the different compartments (chapter 1). In chorionic villi the follow-up sampling rate can be reduced to a minimum when both villi compartments (STC + LTC-villi) are investigated. Since both villi compartments were investigated in our laboratory, the follow-up sampling rate declined to 1.2% in 2000.

Regarding the question 'why did chorionic villus sampling did not reach the popularity of amniocentesis', it is obvious that there are two sides regarding this question:

- From a laboratory point of view it has become clear that most diagnostic pitfalls encountered in chorionic villus sampling can now be avoided in the light of the knowledge and expertise in our department.
- The other side is that the parents decide whether they request first- or second trimester prenatal diagnosis. The parents are informed about the options of prenatal diagnosis and about the advantages and disadvantages of the different techniques.

In our opinion, amniocentesis is to be preferred over chorionic villus sampling at low (cytogenetic) risks (< 3%) but chorionic villus sampling should be the first choice at high (cytogenetic) risks (≥ 3%).

7.2 Future prospects

It is known that procedure related risks have a great impact not only on the decision to choose between chorionic villi and amniocentesis but also on the decision whether to choose prenatal diagnosis or not. In the last two decades different lines of research have been followed to find a screening test to identify women with an increased risk or even to find an alternative test for invasive prenatal diagnosis.

The first test which could identify women at an increased risk for neural tube defects and Down syndrome was the so-called triple test (Wald et al., 1988). It concerns the measurements of AFP, hCG and unconjugated oestriol in maternal serum in the second trimester of pregnancy. Important disadvantages are a detection rate of only 70% and external factors which influence the levels of the markers tested (Chuckle et al., 1990; Wald et al., 1992; Dar et al., 1995; Neveux et al., 1996), the high percentages of false-positives and the relatively late and narrow period of gestation during which the tests must be performed. The definite diagnosis must be made by amniocentesis. As a result, many women become worried unnecessary and in case of an abnormal fetus, termination of pregnancy cannot be offered before 18-19 weeks of gestation. In The Netherlands, for a long period the negative aspects prevailed but, recently, the Health Council of The Netherlands advised the government to implement the triple test on a national scale.

Due to improvements of ultrasound resolution, measurement of the nuchal translucency (NT) thickness between 10-14 weeks of gestation is shown to be a useful marker for fetal aneuploidy. Several reports have demonstrated a link between increased thickness of NT and chromosomal abnormalities, structural and cardiac defects and other syndromes (Brambati et al., 1995; Pandya et al., 1995; Snijders et al., 1998). For women who are initially afraid of invasive prenatal diagnosis because of the procedure related risks, this could be a more attractive alternative than triple test screening in the second trimester. It is noteworthy, that the combination of measurements of serum markers (free β hCG, PAPP A) in the first trimester of pregnancy and ultrasound NT thickness may lead to a better risk estimation than the measurement of NT thickness alone (Noble et al., 1995; de Graaf, 1999). However, the limitations of screening tests, false-positive and maybe more important, false-negative results should be explained to the women involved. However, there is a fair risk that this complex information will not be well understood. The same is true for the triple test, so the expectations of pregnant women embarking on screening may be quite different from reality. Again, the definitive diagnosis must be made by an invasive test. Only, with one major advantage that this can be made by chorionic villus sampling.

For several decades, fetal cells isolated from maternal blood seemed to be a promising alternative to non-invasive prenatal diagnosis. This approach avoids the risks associated with invasive techniques and might provide a definitive diagnosis instead of a risk estimation. Various attempts of isolating fetal nucleated cells from maternal blood samples have been carried out but is restricted by the low frequency of these cells in maternal blood, requiring extensive enrichment and purification procedures (Lo et al.,

1989; Bianchi et al., 1990; Jansen, 2000). For an overview of isolation approaches to recover fetal cells from the maternal circulation and for an overview of cell markers used for the enrichment of fetal nucleated blood cells and depletion of maternal cells, see Jansen, 2000. However, after many years of investment, this non-invasive diagnostic method has not developed into a routine method for prenatal diagnosis.

Maybe more promising is the discovery of the existence of fetal DNA in maternal plasma by Lo et al. in 1997. Fetal DNA has been identified in the plasma of pregnant women at concentrations higher than in the cellular fraction, which may have new implications for non-invasive prenatal diagnosis (Lo et al., 1997; Poon et al., 2000). Furthermore, the detection of fetal DNA in maternal plasma does not require prior enrichment of fetal cells and is much simpler than detecting the few nucleated cells in maternal blood. This new approach has already been used for the prenatal diagnosis of fetal Rhesus D status and fetal gender by PCR (Lo et al., 1998; Zongh et al., 2000). It has also been shown that the amount of fetal DNA is elevated in pregnancies with aneuploidies and that the prenatal detection of trisomy 21 can be accomplished by FISH analysis (Lo et al., 1999; Poon et al., 2000; Van Wijk et al., 2000). At this moment, all reports seems to illustrate the potential applicability of maternal plasma DNA analysis for the prenatal diagnosis of a wide variety of disorders. Although the present results are promising, further investigations and large-scale trials are needed to assess the diagnostic accuracy of this new approach.

In the meantime, we must be aware of the fact that these new approaches will probably never replace the invasive tests completely. Therefore, we must continue with the refinement and development of existing additional techniques, such as FISH, comparative genomic hybridization (CGH) and spectral karyotyping (SKY), and possibly the use of DNA microarrays in the future.

The advent of human telomere probes is a good example of such development (Flint et al., 1995; Knight et al., 2000). However, refinement of the FISH protocols are still necessary to achieve a higher hybridization efficiency needed for prenatal diagnostic applications at metaphase as well as interphase level. Another molecular cytogenetic technique for the identification of chromosomal aberration is SKY-FISH, a technique widely used in tumor cytogenetics (Veldman et al., 1997; Nordgren et al., 2001), also available for chromosomal aberrations of unknown origin in prenatal cytogenetics.
Similarly, high resolution CGH is a valuable technique for the detection of chromosomal unbalances and has been broadly applied in a variety of malignancies (Pinkel et al., 1998; Kirchhoff et al., 2000). However, at the present time, CGH still has some pitfalls and technical difficulties which makes the technique not suitable yet for prenatal diagnosis. The sensitivity of CGH in detecting gains and losses of DNA sequences is approximately 3-5 Mb. For the detection of events involving regions less than 3 Mb locus by locus techniques are required, called DNA microarray, allowing detection of aberrations at the genomic level less than 100kb (MacBeath et al., 2000; Shoemaker et al., 2001). However, this technique is still in a developmental stage.

Fortunatly, it has been shown that prenatal diagnosis can be performed with a high quality and reliability.

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Summary

This thesis presents investigations on the quality and reliability of prenatal cytogenetic diagnosis in amniotic fluid cells and chorionic villi. Amniocentesis and chorionic villus sampling are the two routinely used techniques for the sampling of fetal material for prenatal laboratory investigations. These investigations include DNA analysis, biochemical studies, and cytogenetics (karyotyping and fluorescence in situ hybridization).

In chapter 1, a short overall picture is given on the history of prenatal diagnosis. Possible explanations and answers are given on raised questions why first-trimester chorionic villus sampling has not reached the popularity of second-trimester amniocentesis for cytogenetic investigation. The attention is focussed on laboratory related problems; 1) chromosome quality; 2) laboratory failures and 3) discrepancies in karyotypes between prenatal samples and fetus. The latter and most serious one is discussed in detail. Attention is given to the arising of mosaicism in the early embryo and the subsequent distribution of abnormal and normal cells among the various embryonic compartments. Work-up protocols for the differentiation between pseudomosaicism and mosaicism in AF-cells and for the differentiation between generalized mosaicism and confined placental mosaicism in chorionic villi are given in this chapter.

In chapter 2, the results of abnormal karyotypes in semi-direct (short-term culture; STC) villi are presented. Among 3499 cytogenetically investigated STC-villi, during the years 1993-1996, 219 abnormal karyotypes were encountered. These abnormal karyotypes were devided into two groups; certainly abnormal (n=110) and uncertainly abnormal (n=109). The latter required further investigation. The certainty rates and predictive values of abnormal cytogenetic results of STC-villi (certainly abnormal) among the various indications showed that the cytogenetic performance is only acceptable when the cytogenetic risk is substantially increased (≥3%). This was the case in women with a maternal age of 40 years or more, carriers of structural rearrangements and women displaying fetal abnormalities on ultrasound. Because of the high rate of prenatal follow-up investigations after the finding of uncertain results in STC-villi, recommendations towards the simultaneous analysis of STC-villi and cultured (long-term culture; LTC) villi were made. Furthermore, chorionic villus sampling for cytogenetic investigation was only advised to women with singleton pregnancies having cytogenetic risks equal to or exceeding that of a 40 year old pregnant woman, and to women with multiple pregnancies at any risk.

In chapter 3, cytogenetic results are reported in detail of 1838 consecutive chorionic villus samples, during the years 1997-1999. We investigated whether the recommendations made towards the desirability of the analysis of both STC- and LTC-villi really resulted in; 1) improvement of chromosome quality by the investigation of LTC-villi; 2) a decrease in the rate of uncertain cytogenetic results with a concomitant decrease in the rate of follow-up investigations. The routine analysis of 16 cells was maintained but was devided among STC- and LTC-villi and well adjusted in various circumstances. Cytogenetic investigations in both villi compartments could be made in almost 85%. In just over 15% of the cases, LTC-villi

(91.4%) and abnormal karyotypes were seen in 158 (8.6%) cases divided into 6 categories. Finally only 34 of these cases (21.5%) turned out to be uncertain.
The improvement of karyotype quality was evidently shown in this chapter. The investigation of both villi compartments showed a high degree of laboratory success and reduced follow-up amniocenteses with one-third in comparison with the analysis of STC-villi alone. We believe and conclude that the desired level of quality and accuracy of prenatal cytogenetics in chorionic villi can only be achieved when both STC- and LTC-villi are available.
In chapter 4, amniocentesis and chorionic villus sampling are compared in multiple pregnancies. The laboratory aspects and the consequences of discordant laboratory findings are evaluated in 500 women with multiple gestations (482 twins and 18 triplets). For this study the patients were divided into four groups; twin pregnancies with 2 chorionic villus samples; twin pregnancies with two amniocenteses; twin pregnancies with only one sample or two different samples and the last group comprised the triplet pregnancies. One uncertainly concordant result and six uncertainly discordant results were achieved in seven pregnancies after chorionic villus sampling, and one uncertainly concordant result after amniocentesis. Follow-up investigations were needed in these cases to come to certain results. Finally normal results were seen in 442 pregnancies (88.4%) and abnormal results in 58 pregnancies (11.6%).
Comparing amniocentesis and chorionic villus sampling in multiple gestations allowed the following conclusions; 1) there is a greater risk of uncertain results in chorionic villus sampling, which can be minimized by the simultaneous investigation of STC- and LTC-villi; 2) there is a substantial risk of cross contamination in chorionic villus sampling which can cause problems especially in prenatal biochemical investigations; 3) there is a better performance of selective reduction after chorionic villus sampling than after amniocentesis.
In chapter 5, the diagnostic performances of cytogenetic investigation in AF cells and chorionic villi are compared in order to answer the question when to chose or recommend amniocentesis or chorionic villus sampling. Over a period of seven years (1993-1999) 11.883 AF samples and 5328 chorionic villus samples were investigated in our department. Laboratory failure rate (proportion of appropriate samples without results), karyotype quality (G-band score, rate of follow-up samples, rate of wrong diagnoses) and karyotype representativity (rate of follow-up samples, rate of wrong diagnoses) were measured. The results of this study pointed out that the laboratory failure rate was the same after amniocentesis (0.40%) and chorionic villus sampling (0.50%). The G-band scores were equal in AF-cells and LTC-villi but significantly lower in STC-villi. The rates of follow-up sampling because of quality reasons were the same. Those because of representativity reasons differed significantly in the rank order AF-cells (0.10%), (STC + LTC-) villi (1.31%) and STC-villi (1.99%).
This difference has a biological background; a mosaicism in the blastomeres of the early embryo, may lead to unequal distributions among the embryonic compartments of trophoblast, extra-embryonic mesoderm, and fetus proper. AF-cells are derived from the compartment of interest, but chorionic villi, STC- as well as LTC-villi are derived from other compartments than the compartment of interest. However, the ratios of all follow-up samples and uncertain or abnormal results in STC-, and (STC+LTC-) villi at

cytogenetic risks ≥ 3% were equal to that of AF-cells at risks < 3%.
This indicates that, from a laboratory point of view, amniocentesis is to be preferred over chorionic villus sampling at low cytogenetic risks (<3%), especially when only STC-villi are investigated. At cytogenetic risks ≥3%, chorionic villus sampling is to be preferred.
In chapter 6, several recurrent and/or specific problems are presented.
In chapter 6.1, a cytogenetic survey and follow-up studies are presented of eight trisomy 9 cases (full, mosaic, pseudomosaic and CPM). Essential steps for the differentiation between pseudomosaicism and true mosaicism or CPM and generalized mosaicism are shown. FISH demonstrated that a large number of interphase nuclei can be screened quickly for the presence of a trisomic cell line in a subsequent tissue sample. Recommendations for the management in cases with trisomy 9 are made.
In chapter 6.2, the prevalence and distribution of tetraploid metaphases in chorionic villi are evaluated. A protocol for the management of tetraploid metaphases in chorionic villi was developed which resulted in a strongly reduced number of prenatal follow-up investigations.
In chapter 6.3, a case of trisomy 18 caused by isochromosome 18p and 18q formation is presented. The diagnosis was made by standard cytogenetic techniques in AF cells and confirmed by FISH. The most likely hypothesis regarding the formation of these isochromosomes, appeared to be a postzygotic centromere misdivision followed by a non-disjunctional error since no recombination was observed at any of the investigated loci.
In chapter 6.4, a case of 45,X/46,XY mosaicism is documented. A 45,X karyotype was found in STC-villi and a 46,XY karyotype in LTC-villi, with a 45,X/46,XY karyotype in subsequent AF-cells. Extensive follow-up investigations were carried out to gain insight into this cytogenetic inconsistency. This resulted in two theoretical modes of origin based on the embryonic models presenting combinations of different karyotypes in various fetal compartments.
In chapter 7, the conclusions and implications of the studies in the previous chapters are discussed in general. Our results form a detailed documentation of the accuracy and reliability of cytogenetic investigation in STC-, (STC + LTC-) villi and AF-cells. Finally, this well-ordered total performance showed us that from a laboratory point of view amniocentesis is to be preferred over chorionic villus sampling at low (cytogenetic) risks (< 3%) and chorionic villus sampling should be the prenatal test of choice at high (cytogenetic) risks (≥ 3%).

Samenvatting

Dit proefschrift beschrijft onderzoek naar de kwaliteit en de betrouwbaarheid van cytogenetische diagnostiek in vruchtwater en chorionvlokken.
De vruchtwaterpunctie en de vlokkentest zijn de twee routinematig gebruikte technieken voor het verkrijgen van foetaal materiaal ten behoeve van prenataal laboratoriumonderzoek. Prenataal onderzoek omvat DNA-analyse, biochemische studies en cytogenetisch onderzoek (karyotypering en fluorescentie in situ hybridisatie).
Hoofdstuk 1 start met een korte beschrijving van de geschiedenis van de prenatale diagnostiek. Mogelijke verklaringen en antwoorden worden gegeven op de vraag waarom de vlokkentest in het eerste trimester van de zwangerschap nooit de populariteit van de vruchtwaterpunctie in het tweede trimester heeft gehaald.
Aansluitend richt de aandacht zich op laboratorium gerelateerde problemen als: 1) chromosoom kwaliteit, 2) mislukken van het onderzoek; 3) discrepanties tussen het karyotype in het prenataal onderzochte materiaal en de foetus. Dit laatste blijkt het grootste probleem te zijn en hangt samen met het ontstaan van mozaïeken in een vroeg stadium van het embryo en de daarop volgende distributie van normale- en afwijkende cellen over de verschillende embryonale compartimenten.
Het hoofdstuk sluit af met een beschrijving van twee uitgebreide protocollen. Ten eerste voor de differentiatie tussen pseudomozaïeken en een werkelijk mozaïek in vruchtwater cellen en ten tweede voor de differentiatie tussen gegeneraliseerde mozaïeken en mozaïeken beperkt tot de placenta in cellen van de chorionvlokken.
Hoofdstuk 2 richt zich op de resultaten van afwijkende karyotypes in semi-directe vlokken (STC-villi). In een periode van 6 jaar (1993-1999) zijn 3499 STC-villi onderzocht en werden er 219 afwijkende karyotypes gevonden welke in twee groepen werden onderverdeeld; zeker afwijkend (n=110) en onzeker afwijkend (n=109). Voor de laatste groep was vervolgonderzoek noodzakelijk. De voorspellende waarde van de zekere uitslagen verdeeld over de verschillende indicaties scoorde het best bij de hoge risico's (vrouwen van 40 jaar en ouder, dragers van structurele afwijkingen en zwangerschappen met echo afwijkingen).
Naar aanleiding van het hoge aantal vervolgonderzoeken na onzekere uitslagen gevonden in STC-villi, verdiende het aanbeveling om zowel STC-villi als gekweekte vlokken (LTC-villi) te onderzoeken. Tevens kregen vrouwen met een hoog risico, naar aanleiding van deze studie, desgevraagd het advies om een vlokken test te ondergaan in plaats van een vruchtwaterpunctie.
In hoofdstuk 3 wordt een gedetailleerde studie naar de cytogenetische resultaten van 1838 vlokkentetsen (1997 tot en met 1999) gepresenteerd. Deze studie had tot doel te onderzoeken of de in 1996 gedane aanbevelingen om zowel STC-villi als LTC-villi te onderzoeken werkelijk een betere chromosoomkwaliteit opleverde en resulteerde in een afname van het aantal onzekere resultaten.
In deze studie was er in 85% van de vlokkentesten voldoende materiaal om beide compartimenten (STC- + LTC-villi) te onderzoeken. Van de resterende 15% was er niet genoeg materiaal beschikbaar en kon alleen het compartiment van de STC-villi onderzocht worden. In 158 patiënten werden afwijkende karyotypes gevonden (8.6%) welke in zes categorieën werden onderverdeeld. Uiteindelijk bleken slechts 34 uitslagen

(21.5%) werkelijk onzeker te zijn. In dit hoofdstuk zijn ook voorbeelden te zien waar de verbetering van de kwaliteit van chromosomen in LTC-villi duidelijk blijkt. Samen met het hoge percentage zekere uitslagen en de sterke afname van het aantal vervolgonderzoeken kan gesteld worden dat een hoge kwaliteit en betrouwbaarheid alleen bereikt kan worden als zowel de STC- en de LTC-villi worden onderzocht.
Hoofdstuk 4 betreft een studie waarbij de vruchtwaterpunctie en de vlokkentest met elkaar vergeleken worden in geval van meerling-zwangerschappen. Een evaluatie van laboratoriumaspecten en de consequenties van discordante uitslagen van 500 meerling-zwangerschappen is daarbij opgenomen.
Er werd een onderverdeling gemaakt in vier groepen: 1) tweelingen met twee vruchtwaterpuncties; 2) tweelingen met twee vlokkentesten; 3) tweelingen met twee verschillende afnames of slechts één afname; 4) drielingen. In 7 zwangerschappen werden na een vlokkentest onzekere uitslagen gevonden en in 1 na een vruchtwaterpunctie. In alle 8 zwangerschappen werd vervolgonderzoek verricht.
Vruchtwaterpunctie en vlokkentest met elkaar vergelijkend in geval van meerling-zwangerschappen gaf de volgende conclusies. Er is een groter risico op vervolgonderzoek na een vlokkentest. Dit percentage is echter tot een minimum terug te brengen wanneer zowel STC- als LTC-villi wordt onderzocht. Er is een zeker risico op cross-contaminatie bij een vlokkentest wat een probleem bij biochemische bepalingen kan vormen. Er is een geringere kans op het verlies van de gezonde foetus na een selectieve reductie uitgevoerd na de vlokkentest dan na de vruchtwaterpunctie.
In hoofdstuk 5 wordt de diagnostische kwaliteit van cytogenetisch onderzoek na een vruchtwaterpunctie vergeleken met die na de vlokkentest met als doel een antwoord te kunnen geven op de vraag of en wanneer de vruchtwaterpunctie dan wel de vlokkentest de voorkeur geniet. Gedurende een periode van zeven jaar (1993-1999) zijn er 11.883 vruchtwatermonsters en 5328 vlokkenmonsters onderzocht.
Het percentage van mislukte onderzoeken, de kwaliteit en de representativiteit van de karyotypen werden daarbij gemeten.
Het aantal mislukte onderzoeken bij beide bleek erg laag (0.5%). De kwaliteit van de chromosomen van vruchtwatercellen en LTC-villi was gelijk, terwijl de kwaliteit in de STC-villi beduidend minder was.
Het aantal vervolgonderzoeken om kwaliteitsredenen was ook gelijk. Het aantal vervolgonderzoeken als gevolg van representativiteit echter, was bij STC-villi het hoogst en bij vruchtwater het laagst. Hieraan ligt een biologische oorzaak ten grondslag. Een mozaïek in de blastomeren van een jong embryo kan leiden tot een ongelijke verdeling van afwijkende en normale cellen tussen de verschillende embryonale compartimenten; de trophoblast cellen (STC-villi), cellen van het extra embryonale mesoderm (LTC-villi) en cellen van de toekomstige foetus (vruchtwatercellen).
Deze studie liet wel zien dat de verhouding van het totaal aantal vervolgonderzoeken, in relatie tot onzekere en zeker- afwijkende uitslagen na de vlokkentest bij hoge risico's (≥3%) gelijk was aan die na de vruchtwaterpunctie bij lage risico's (< 3%). Vanuit laboratorium oogpunt gaat de voorkeur uit naar een vruchtwaterpunctie waar het lage risico's betreft en naar de vlokkentest bij hoge risicogroepen (≥ 3%).
Tijdens de eerder beschreven studies deden zich regelmatig terugkerende specifieke problemen voor welke in hoofdstuk 6 onder de loep zijn genomen.

In hoofdstuk 6.1 wordt een overzicht gegeven van acht casussen met of een volledige trisomie 9, een mozaïek trisomie 9, een pseudomozaïek of een mozaïek beperkt tot de placenta. Centraal hierbij staan de te nemen essentiële stappen om onderscheid te kunnen maken tussen de verschillende mozaïeken en het gebruik van FISH.
Hoofdstuk 6.2 betreft een evaluatie met betrekking tot het voorkomen van tetraploide metafases in chorionvlokken. Aansluitend wordt een werkprotocol geïntroduceerd om in geval van tetraploidie mozaïeken het aantal vervolgonderzoeken terug te dringen met behoud van diagnostische accuraatheid.
Het voorkomen van isochromosomen en een hypothese over het ontstaans-mechanisme van isochromosoom 18p en 18q wordt in hoofdstuk 6.3 besproken.
In hoofdstuk 6.4 wordt een casus besproken met een mozaïek 45,X/46,XY. Na de vlokkentest werd een 45,X karyotype in STC-villi een 46,XY karyotype in LTC-villi. Vervolgonderzoek in vruchtwatercellen liet een mozaiek 45,X/46,XY zien. Ook hier werd door middel van uitgebreid follow up onderzoek een verklaring in het ontstaans-mechanisme neergelegd.
Tot slot worden in hoofdstuk 7 de conclusies en implicaties van alle hoofdstukken bediscussieerd. Alle resultaten vormen een zeer gedetailleerde documentatie over de accuraatheid en betrouwbaarheid van prenataal cytogenetisch onderzoek middels vruchtwaterpunctie en vlokkentest.
Uit de in dit proefschrift beschreven resultaten kan worden afgeleid dat, vanuit laboratorium oogpunt gezien, een vruchtwaterpunctie wordt verkozen boven een vlokkentest als het gaat om de lage risico's (<3%) en dat bij de hoge risico's (≥3%) een vlokkentest geadviseerd en verkozen wordt boven een vruchtwaterpunctie.

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1974	MAVO-4, 's-Gravenhage	
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Wanneer je een grote verre reis gaat maken dan komt daar veel voor kijken. Als je bijvoorbeeld een trekking gaat doen moet er behalve visum, permit en gidsen nog veel meer geregeld worden zoals; hulpgids(en), dragers, kok en keukenhulpen. Zo komt er bij het werken aan een proefschrift ook veel kijken. Dus, naast de eerder genoemde gidsen gaat mijn dank ook uit naar al degene die meehielpen mijn reis te voltooien en mijn reisverslag of een deel daarvan met kritische blik hebben gelezen en van commentaar hebben voorzien. Ik denk daarbij natuurlijk aan de leden van de kleine commissie die ik erkentelijk ben voor het snelle beoordelen van mijn boekje.

Daar ik al heel wat jaren op de prenatale diagnostiek werk heb ik met heel veel mensen samengewerkt. Zou ik deze met naam en toename gaan noemen dan zou ik zeker mensen vergeten of te kort doen.

De medewerkers van de 24ste, met name die van postnataal, biochemie en de medewerkers van de sector prenatale diagnostiek van Dijkzigt en Dordt wil ik bedanken voor hun prettige samenwerking.
Het DNA lab wil ik danken voor hun gastvrijheid en de inspanningen die ze geleverd hebben om mij wegwijs te maken in enkele DNA technieken. Datgene wat ik bij jullie geleerd heb is ook in dit boekje terug te vinden.
Natuurlijk gaat mijn dank ook uit naar al die medewerkers/collega's die er steeds weer voor zorgen dat het lab optimaal kan functioneren. Dank voor al het steriele glaswerk, de foto's , bestellingen en niet te vergeten het secretariële werk.

Bij het schrijven van dit dankwoord heb ik er voor gekozen om eigenlijk zo min mogelijk namen te noemen om te voorkomen dat ik iemand of vergeet of te kort doe. Toch valt dat niet mee wanneer ik uiteindelijk bij de belangrijkste crew van mijn tocht beland en dat zijn de harde werkers van het prenatale lab. Tegen al mijn collega's van het prenatale diagnostiek lab durf ik met enige trots het volgende te zeggen: "Jullie inzet en betrokkenheid vormen de kern en de kracht van de afdeling". Het feit dat hier een team werkt heeft mij in de gelegenheid gesteld om mijn aandacht te verdelen tussen mijn dagelijkse werkzaamheden en het schrijven van artikelen en het afronden van dit proefschrift. Mijn dank is niet alleen voor jullie keiharde werken en jullie hulp maar ook voor jullie begrip, niet alleen voor mij maar ook voor elkaar.

Voor mijn twee paranimfen, Armando en Diane, wil ik net als mijn drie gidsen een uitzondering maken in de vorm van een persoonlijke noot.
Armando, ik ben blij dat je mijn paranimf wil zijn (en blijven). Jouw inzet en betrokkenheid voor het lab is niet alleen voor mij maar voor het hele lab van onschatbare waarde geweest. Je liefde voor computers heeft het uiteindelijk gewonnen en ik wens je dan ook veel succes bij je nieuwe uitdaging.
Diane, het feit dat jij vanaf het moment dat ik het je vertelde, heel enthousiast en positief met mijn promotie plannen bent omgegaan heeft mij meer dan goed gedaan. Ik ben blij dat we goede collega's zijn en dat ik op mijn beurt veel van je kan leren. Ik hoop dan ook dat we nog lang en met heel veel plezier en wederzijds respect collega's zullen blijven.

Het leven buiten het lab speelt natuurlijk ook een grote rol en vandaar dat ik de laatste alinea's aan familie en vrienden wil wijden. Lieve mensen, de aandacht en interesse in mijn voortgang van mijn proefschrift die ik van jullie kreeg hebben mij veel goed gedaan. Tijdens alle etentjes, borrels, weekendjes Ardennen, zeil- en kanotochtjes, weekjes Verdon, en andere uitstapjes kreeg ik altijd jullie aandacht. Dit heeft mij vaak weer een stukje extra energie gegeven op die momenten waar ik het harder nodig had dan menigeen besefte. Ik ben jullie daar veel dank voor verschuldigd. Hopelijk gaan we weer snel wat ondernemen!

Tijdens de tocht van het promoveren heb ik natuurlijk veel aan het thuisfront gedacht. Immers, dank zij hen kreeg ik het vermogen en de vrijheid om zo'n belangrijke tocht als deze te ondernemen.
Lieve pa en ma, ik weet dat jullie trots op mij zijn maar misschien ben ik nog wel trotser op jullie, want voor een heel groot deel ben ik dankzij jullie diegene die ik nu ben. Het doorzettingsvermogen, zeker in moeilijke tijden, is een goede voorbeeldfunctie geweest waar ik veel aan heb gehad en nog steeds heb.
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Stellingen

behorend bij het proefschrift

Quality and Reliability of Prenatal Cytogenetics

1. Cytogenetische discrepanties tussen chorion vlokken en foetus zijn in principe te verklaren door de distributie van cellen over de verschillende embryonale compartimenten.
Dit proefschrift

2. Een betrouwbare cytogenetische diagnose in chorion vlokken kan alleen worden gesteld wanneer zowel cytotrophoblast-cellen als cellen uit de mesenchymale kern zijn onderzocht.
Dit proefschrift

3. Bij monochoriale biamniotische tweelingen is het van belang om in geval van een vlokkentest beide foetus en beide compartimenten van de vlokken te onderzoeken.
Dit proefschrift

4. Bij meerlingzwangerschappen verdient de vlokkentest de voorkeur boven de vruchtwaterpunctie.
Dit proefschrift

5. De afname van het aantal vlokkentesten ten opzichte van het aantal vruchtwaterpuncties is eerder te verklaren door een selectief gebruik dan door beperkingen van de vlokkentest.
Dit proefschrift

6. Het inbrengen van mitochondriaal DNA van een jonge donorvrouw in eicellen van onvruchtbare vrouwen lijkt technisch veelbelovend ware het niet dat deze ooplasma transfer ook erfelijke ziektes kan introduceren.

7. Als serumscreening ook gebruikt zou kunnen worden om de juistheid van een politiek besluit te bepalen, zou deze test nooit door de Kamer worden aangenomen.

8. Het ontrafelen van het menselijk genoom is een voorbeeld van het vinden van antwoorden op nog onbekende vragen.

9. Het begrip wetenschap suggereert dat kennis op een plank ligt.

10. Naarmate de belijders van een geloof sterker georganiseerd raken, neemt de mate van tolerantie af.

11. Als je kennis wilt vermenigvuldigen, moet je bereid zijn haar te delen.

Rotterdam, 9 januari 2002

Cardi van den Berg