Quality assessment of prenatal cytogenetic diagnosis

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Chapter 4

Chromosomal analysis in preparations from chorionic villi and amniotic fluid cell cultures

Chapter 4.1

A 46,XX,der(13;14)(q10;q10),+21 child born after a 45,der(13;14)(q10;q10) chromosomal finding in CVS

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Letter to the Editor:

A 38- year- old gravida 5, para 2 woman underwent chorionic villus sampling (CVS) at 10+3 weeks of gestation because she is a carrier of a Robertsonian translocation (13;14). Chromosome analysis was performed after direct preparation. A female chromosome pattern with a Robertsonian translocation 45,XX, der(13;14)(q10;q10) was found in 15 GTG-banded metaphases.

The pregnancy continued uncomplicated and a female infant with clinical manifestations of Down syndrome was born spontaneously at 38 weeks. Cytogenetic analyses of PHA- stimulated lymphocyte culture showed 46,XX, der (13;14)(q10;q10), +21 (5 metaphases). Fluorescence *in situ* hybridisation (FISH) with the probe YAC259H11 showed 3 signals for chromosome 21 in 100 metaphases.

Slides of the direct chorionic villus preparation were subsequently re-examined. An additional 112 metaphases were seen, all without a trisomy 21. There was no chorionic villus culture available.

Discordant findings between CVS and the foetal tissue have been documented before. The majority of the reported cases have been false-positive results (CVS abnormal, child normal). In a very few instances, a false-negative result (CVS normal, child abnormal) has been reported. There are several mechanisms by which the false-negative results may have arisen, including:

- a post-zygotic non-disjunction in the inner cell mass during embryogenesis (Crane and Cheung, 1988);

- a twin pregnancy, with loss and re-absorption of the normal foetus;

- a mosaic situation, undetected because of the low mitotic index; and

- contamination of the villi with normal maternal cells (Pindar et al.,1992).

In 1987 Simoni et al. made a rough estimation about the incidence (1 per thousand) of a false-negative result. Lilford et al. (1991) suggest a frequency of

0.001per cent using the (semi)direct method. The case described here is the first false-negative result in our series of 7000 CVS patients by the direct and 1000 patients by the culture method.

Table 1: False-negative results in CVS for sex-chromosomal abnormalities

Foetus	Direct CVS	Culture	Indication	Reference
Fibroblasts:				
47,XYY[23]/46,XY[39]	46,XY[85]	47,XXY[43]/46,XY[7]	?	Callen et al. (1988)
Blood:				
47,XXY[20]/46,XY[58]	46,XY[85]	47,XXY[58]/46,XY[7]	Maternal age	Eichenbaum et al. (1986)
Blood:				
47,XXY[?]/46,XY[?]	46,XY[10]	47,XXY[?]	Maternal age	Linton and Lilford (1986)
Blood:				
47,XXY[7]/46,XY[13]	46,XY[15]	47,XXY[10]/46,XY[5]	?	Miny et al. (1991)
Amniotic fluid:				
45,X[28]/46,XY[32]	46,XY[12]	45,X[21]/46,XY[5]	Maternal age	Smidt-Jensen and Lind (1987)
Different tissues:				
45,X[9]/46,XX[281]	46,XX[50]	45,X[50]	Previous child +21	Schlesinger et al. (1990)
Blood:				
47,XXX[47]/46,XX[3]	46,XX[35]	---	Maternal age	Verjaal et al. (1987)

Table 2 – False-negative results in CVS for trisomies 18 and 21

Fetus	Direct CVS	Culture	Indication	Reference
Different tissues:				
47,XY, + 18[47]	46,XY[37]	47,XY, + 18[30]	Maternal age	Martin et al. (1986)
Amniotic fluid:				
47,XY, + 18[9]	46,XY[15]	47,XY, + 18[5]	?	Miny et al. (1991)
Blood:				
47,XX, + 18[10]/46,XX[2]	46,XX[10]	47,XX, + 18[15]/46,XX[15]	US abnormalities at 30 weeks	Leschot et al. (1988)
Blood:				
47,XX, + 18[56]	46,XX[16]	–	US abnormalities at 31 weeks	Wirtz et al. (1988)
Fibroblasts:				
47,XX, + 18[30]	45,XX[4]	46,XX[11]	Maternal age	Pindar et al. (1992)
Blood:				
47,XY, + 21[99]	46,XY[50]	–	Maternal age	Bartels et al. (1989)
Amniotic fluid:				
47,XY, + 21[15]/46,XY[72]	46,XY[25]	47,XY, + 21[90]/46,XY[10]	?	Callen et al. (1988)
Different tissues:				
47,XY, + 21[300]	46,XY[110]	47,XY, + 21[100]	Maternal age	Lilford et al. (1991)
Amniotic fluid:				
47,XX, + 21[12]/46,XX[24]	46,XX[25]	47,XX, + 21[11]	Maternal age	Miny et al. (1988)
Blood:				
47,XX, + 21[6]/46,XX[94]	46,XX[100]	47,XX, + 21[8]/46,XX[24]	Previous child + 21	Nisani et al. (1989)
Blood:				
47,XX, + 21[17]	46,XX[20]	–	Maternal age	Simoni et al. (1987)
Amniotic fluid:				
47,XX, + 21[?]	46,XX[?]	47,XX, + 21[?]	?	Vejerslev & Mikkelsen (1989)
Blood:				
46,XX,der(13;14),+ 21[105]	45,XX,der(13;14)[129]	–	Mother der(13;14)	Presented case (1997)

To our knowledge, 20 cases (excluding structural abnormalities) of a false-negative finding including our case have been published: seven cases with a sex-chromosomal abnormality (Table 1); five cases with trisomy 18; and eight cases with trisomy 21 (Table 2). In five cases, the (semi)direct method only was performed. In most of the cases (11 times), the culture method gave the right result whereas the (semi)direct method gave a false-negative result. In three cases, the (semi)direct method was normal, the culture was non-mosaic abnormal, and chromosome studies of the blood of the child showed a mosaic sex- chromosomal abnormality. In the case reported by Pindar et al. (1992), the (semi)direct as well as the culture method gave a false-negative result (46,XX), whereas the child had a 47,XX,+18 chromosomal pattern. By this new case it is again demonstrated that the most favourable technique for accurate prediction is the combination of the (semi)direct and culture method. According to these data, the culture method alone is a very good second choice.

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Chapter 4.2

Four years' cytogenetic experience with the culture of chorionic villi

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4.2.1 Summary

In 1958 chorionic villus samples, investigated by culture method, we found 137 (7%) abnormalities. The abnormal results were classified in certain abnormal (generalised abnormal at high probability) and uncertain abnormal (potentially confined to the placenta) results. Certain abnormal were 73 cases (3.7%). Uncertain abnormal were 64 cases (3.3%), in which confirmation studies were done in 47 cases. In 12 cases of these 47, the abnormality was confirmed and in 35 cases (1.8%) the abnormality was confined to the placenta. Among the latter cases, poor pregnancy outcome [(16% intrauterine death (IUD), 6% intrauterine growth retardation (IUGR)] was increased. Total maternal cell contamination was not seen.

The positive predictive value of all confirmed abnormal cases was 66%. The positive predictive value was 100% for indications 'ultrasound abnormalities' and 'carrier' and between 50 and 60% for all other indications. Predictive value among uncertain abnormal cases was low (26%). However, the positive predictive value depends of the type of abnormality. Therefore we conclude that the culture method for chorionic villi is a good test for indications 'ultrasound abnormalities' and 'carrier' and reliable for all other indications. Whether or not follow-up investigations should be offered to the parents depends of the type of abnormality. We conclude that the culture method is reliable for prenatal diagnosis and can be used as the sole investigated method.

Key words:

chorionic villus sampling (CVS); confined placental mosaicism (CPM); culture method; predictive value

4.2.2 Introduction

Chorionic villus sampling (CVS) is widely used as a method for the prenatal detection of chromosome abnormalities. Two approaches were developed for cytogenetic diagnosis: a (semi)direct method (Simoni et al., 1983) and a culture method (Niazi et al., 1981; Heaton et al., 1984). However, soon after the introduction of CVS it transpired that cytogenetic diagnosis did not always reflect the chromosomal constitution of the foetus. In these cases the aberration was confined to the placenta, so-called confined placental mosaicism (CPM) (Kalousek and Dill, 1983). Reports on false positive and false negative results have been published (Brambati et al., 1985; Breed et al., 1986; Eichenbaum et al., 1986).

Various (collaborative) studies (Ledbetter et al., 1992; Association of Clinical Cytogeneticists Working Party on Chorionic Villi in Prenatal Diagnosis, 1994; Hahnemann et al., 1997a; Smith et al., 1999) described cytogenetic findings and pregnancy outcome of the (semi)direct and/ or culture method. These studies reported on CPM (false negative and false positive findings), maternal cell contamination (MCC) and calculations of the predictive value of the method employed. A combination of the (semi)direct and culture method was recommended to achieve the highest predictive value. When using only one method, the culture method should be the method of choice because of fewer false negative and/ or false positive findings (Ledbetter et al., 1992).

Formerly in our laboratory we used the direct method for routine cytogenetic diagnosis. In case of abnormalities, the culture was also investigated (Breed et al.,1990).

Based on the results of collaborative studies and on a false negative result in our laboratory (Sikkema- Raddatz et al., 1997) we decided to change our policy. We skipped the direct method and now use only the culture method, without increasing turn around time of diagnosis. For economic reason it is not possible to use both culture and direct method.

In the present study we describe our results with the culture method over a 4-year period. We describe the CVS success rate, MCC, the aberrations, and calculate the predictive value (false positive results).

4.2.3 Materials and Methods

Between 1994 and 1998 we received 1958 chorionic villus samples. The majority of the samples (1723) came from the University Hospital in Groningen. The other 235 samples came from the hospital "Isala Klinieken" in Zwolle.

CVS was performed transcervically between 10 and 12 weeks of gestation as described previously (Breed et al., 1990). Exceptionally, CVS was performed transabdominally in cases of more than 12 weeks' gestation. Chorionic villi were treated with trypsin and collagenase before being cultured (according to the method of Jackson et al., 1989). The cell suspension was then transferred to coverslips and cultured in Amniomax and Chang with α-mem. After adequate growth, cultures were harvested after 6 days on average. Karyotyping was routinely performed after Pancreatin- Giemsa staining. Other additional staining techniques, such as DA-DAPI-staining, AgNOR- staining, C- banding and FISH were used, for confirming a suspect chromosomal diagnosis after Pancreatin- Giemsa staining or when a rapid diagnosis was required.

At least 15 cells were analysed. This number was extended to 29 cells in case of a single cell with a trisomy 8, 9, 13, 18, 20, 21 or 22, a supernumerary marker chromosome (ESAC) or a single cell with gain or loss of a sex chromosome.

A normal result was given in cases of 46,XX or 46,XY, in case of the common inv(9)(p11q13), in cases of less than 50% of tetraploid cells and/ or in cases of one cell exhibited any abnormality. All other results were considered abnormal.

Abnormal cytogenetic results

Between the abnormal results a distinction was made in non- mosaic cases and mosaic cases.

A cytogenetic result was defined as mosaic when two or more cells showed a karyotype different from the karyotype(s) in the other cells. Mosaic monosomy was considered when three cells all lacked the same chromosome. In cases of a mosaic result the parents were told that further investigation in amniotic fluid was required to evaluate the result in chorionic villi.

The non-mosaic results were classified as structural and numerical abnormal results. In cases of a structural abnormal result parental karyotyping was done, if the structural abnormality was not known to be familial. In cases of a numerical abnormal result further investigation in amniotic fluid was carried out to evaluate the result in chorionic villi. An exception was made in cases of trisomy 21 and in cases of ultrasound abnormalities in combination with a cytogenetic abnormality.

Certain abnormal cytogenetic results

Abnormal results were defined as certain when a non-mosaic trisomy 21, a non-mosaic sex chromosomal trisomy, a *non*-mosaic triploidy, a *non*-mosaic structural abnormality, or a non-mosaic ESAC was found. In addition, a non-mosaic 45,X pattern and a trisomy 18 in combination with ultrasound abnormalities were defined as certain abnormal. Trisomy 13 was not seen in this study, but would have been counselled similar to trisomy 18.

Uncertain abnormal cytogenetic results

Abnormal results were defined as uncertain when a non-mosaic trisomy 18 and a 45,X pattern without ultrasound abnormalities, a non-mosaic autosomal trisomy other than 13, 18 or 21 and all kinds of mosaic patterns (both numerical and structural) were found.

Predictive value

Predictive value was defined as the probability of an abnormal karyotype of the foetus, given that CVS showed either a mosaic and/ or non-mosaic abnormality.

The 95% confidence intervals (CI) of the predictive value (p) with a standard error SE(p) were calculated as follows:

$CI(p)= p \pm 1.96 * SE(p)$ and $SE(p)= \sqrt{p(1- p) / n}$.

Follow-up studies

In cases of uncertain abnormal results, follow-up studies (chromosomal study of (un)cultured amniocytes, parental peripheral blood karyotyping or ultrasound investigations) were offered to the parents.

Amniotic fluid cells were cultured according to the *in situ* method. Clones (n= 29) were analysed to exclude at least 10% of a mosaic pattern (95% CI). In some cases fluorescence *in situ* hybridisation (FISH) was performed on metaphase chromosomes and/or on uncultured amniocytes confirming suspect chromosomal results and/ or for rapid diagnosis. DNA studies were performed to investigate the possibility of uniparental disomy (UPD) in abnormal CVS cases in which chromosome 15 was involved.

Peripheral blood lymphocytes were prepared according to standard techniques for karyotyping.

First and second trimester ultrasound investigations were performed by a gynaecologist subspecialized in prenatal diagnosis with the use of third level equipment (Acuson ^{TM} Corporation, Mountain View, CA, USA, 128xP10).

In cases of termination of pregnancy (TOP) we always tried to confirm the cytogenetic diagnosis in foetal fibroblasts. Information about the course and outcome of pregnancy was received from the woman and/or the referring midwife or physician. Birth weights of babies were categorised according to the tables of Kloosterman (1970).

4.2.4 Results

From the 1958 samples, a successful diagnosis was obtained in 99,7%. Of the six failures, four involved small samples (≤5 mg). In one case cells did not attach on coverslips. In the final case cell growth was very poor; only two cells could be examined.

Normal karyotypes were found in 1815 cases. Follow-up revealed no false negative results. Abnormal karyotypes were found in 137 cases (7%) [Table 1]. Single cell abnormalities were counted in 362 cases (18.5%).

Certain abnormal cytogenetic results

A total of 73 cases (3.7%) were certain abnormal (Table 2), including 34 cases of structural abnormalities and 39 cases of numerical abnormalities. Confirmation studies of the 73 certain abnormal cases were done in 58 cases.

Uncertain abnormal cytogenetic results

A total of 64 cases (3.3%) were uncertain abnormal (Table 3): 16 non-mosaic and 48 mosaic cases (2.45%). Confirmation studies were done in 47 cases. In 12 of these cases the abnormality was confirmed. Thus of the 47 cases of expected CPM, 35 cases (1.8%) were indeed confined to the placenta. Not confirmed were two cases of trisomy 16 and 2 cases with a 45,X. In one of the 45,X cases, a 46,XX pattern was found in foetal tissue. Maternal contamination could not be excluded entirely in this tissue. In the other case, a mos 45,X/46,XX pattern was found in foetal tissue after TOP. In the confirmation studies of the mosaic abnormalities, 3/5 cases with a mosaic sex chromosomal abnormality and 1/4 cases with a mosaic ESAC were confirmed.

Table 1 – Number of abnormal results for different indications

			Non-mosaic cases									Mosaic cases					
									Structural rearrangements			Autosomal trisomy					
Indication	Number of cases	Normal cases	Trisomy 21	47,XXX 47,XXY 47,XYY	69,XXX 69,XXY 69,XYY	Trisomy 18	45,X	Unusual trisomy	Balanced	Unbalanced	ESAC	13;18;21	Other	Sex-chromosomal	Structural	ESAC	Tetraploid
MA 36-39	1212	1154	9	3	1	4	3	2	8	1	2	1	12	5	4	1	2
MA > 40	373	342	8	1		4			3			3	5		2	3	2
US abn[a]	47	32	5		6	1	2			1							
Carrier[b]	33	14	1						13	2				1	1	1	
DNA/BIO [c]	114	110					1		1				1				1
Other[d]	173	163	2				2		3				1		1	1	
All	**1952**	**1815**	**25**	**4**	**7**	**9**	**8**	**2**	**28**	**4**	**2**	**4**	**19**	**6**	**8**	**6**	**5**

[a]Fetal abnormalities at ultrasound.
[b]Parental carriership for structural rearrangement.
[c]Cytogenetic investigation secondary to DNA or biochemical investigation.
[d]Previous child with chromosomal abnormality or other reasons for prenatal cytogenetic diagnosis.
MA, Maternal age (years).

Predictive value

Of all 137 abnormal cases, confirmation studies were done in 105 cases. Of the 105 cases (58 certain and 47 uncertain abnormal cases) the abnormality was confirmed in 70 cases. Therefore predictive value for all confirmed cases was 66% (95% CI: 57-75%) (Table 4).

The predictive value of the 58 certain abnormal cases was 100%. The predictive value of the 47 uncertain abnormal cases was 26% (95 % CI: 14%- 38%). Predictive values differed for different abnormalities (Table 3).

Predictive values for different indications are shown in Table 4. The indication 'carrier' resulted in nearly 100% because of one case of a non-mosaic inv(7) in combination with a mosaic +inv(7) pattern. The mosaic +inv(7) was confined to the placenta and not confirmed in amniotic fluid.

Maternal cell contamination (MCC)

There was no case of incorrect sex prediction. In 71 cases (3.6%) a XX/XY admixture, an indication of MCC, was found. All male foetuses were carefully screened for XX-cells (on average 100 metaphases). In most of these cases (n=51) less than ten cells of MCC were seen. In 16 cases MCC was between 10 and 50%. It was remarkable that this high percentage was seen in only one of the two culture systems in eight cases. The other culture system was without MCC. In four cases MCC was > 50%, in one case it was up to 70%.

Table 2 – Certain abnormal results (generalised abnormal at high probability)

Abnormality	Number of cases (%)	Abnormality confirmed/ number of confirmation studies	Positive predictive value (%)
Autosomal aneuploidy			
Trisomy 21	25 (1.3)	14/14	100
Trisomy 18	1 (0.05)	1/1	100
Sex chromosomal aneuploidy			
45,X	2 (0.09)		
47,XXX	2 (0.09)	1/1	100
47,XXY	2 (0.09)	2/2	100
Triploidy	7 (0.36)	6/6	100
Structural abnormality			
Familial balanced	13 (0.7)	13/13	100
Familial unbalanced	2 (0.09)	2/2	100
Possible *de novo*			
Balanced	15 (0.8)	15/15	100
Unbalanced	2 (0.09)	2/2	100
ESAC	2 (0.09)	2/2	100
Total	**73 (3.7)**	**58/58**	**100**

Table 3 – Uncertain abnormal results (potentially confined to the placenta)

Abnormality	Number of cases (%)	Abnormality confirmed/ number of confirmation studies	Positive predictive value (%)	95% confidence
Non-mosaics				
Autosomal				
Trisomy 18	8 (0.4)	6/6	100	20-100%
Trisomy 16	2 (0.1)	0/2	0	
Sex chromosomal				
45,X	6 (0.3)	2/4	50	1-99%
Mosaics				
Autosomal	23 (1.2)	0/19	0	
Sex chromosomal	6 (0.3)	3/5	60	13-100%
Tetraploidy	5 (0.3)	0/3	0	
Structural	14 (0.7)	1/8	12.50	0-35.5%
Total	**64 (3.3)**	**12/47**	**26**	**13.5-38.5%**

Table 4 – Positive predictive value of all confirmed cases for different indications

Indication	Number of cases	Number of abnormal cases (%)	Abnormality confirmed/ number of confirmation studies	Positive predictive value (%) (95% CI[e])
MA 36 ≥39	1212	58 (4.79)	27/49	55 (41-69%)
MA ≥40	373	31 (8.33)	10/19	53 (31-78%)
US abnormalities[a]	47	15 (31.91)	11/11	100 (41-100%)
Carrier[b]	33	19 (57.58)	17/18	94 (83-100%)
DNA/BIO [c]	114	5 (4.51)	2/3	66 (12-100%)
Other[d]	173	10 (5.78)	3/5	60 (17-100%)
Total	**1952**	**137 (7.00)**	**70/105**	**66 (57-75%)**

[a]Fetal abnormalities at ultrasound.
[b]Parental carriership for structural rearrangement.
[c]Cytogenetic investigation secondary to DNA or biochemical investigation.
[d]Previous child with chromosomal abnormality or other reasons for prenatal cytogenetic diagnosis.
[e]95% Confidence interval.
MA, Maternal age (years).

Pregnancy outcome of uncertain abnormal cytogenetic results

Of the 64 uncertain abnormal cases confirmation studies were done in 47 cases. In 35 cases the abnormality was not confirmed in cytogenetic follow-up studies. Of these cases 26 (74%) had a normal pregnancy outcome, four (11%) ended in an intra uterine death (IUD), one pregnancy (3%) was terminated, three (9%) had intrauterine growth retardation (IUGR) and one (3%) developed HELLP (haemolysis, elevated liver enzymes, low platelet count) syndrome. The cytogenetic results of the four pregnancies that ended in IUD were 45,X, trisomy 16, a mosaic tetraploidy and a mosaic trisomy 7. The cytogenetic results of the three pregnancies with IUGR were a mosaic trisomy 2, a mosaic trisomy 21 and a mosaic 45X/46,XX.

4.2.5 Discussion

This study confirms the results of previous studies in terms of cytogenetic findings and pregnancy outcome. The negative predictive value is 100% since we had no cases with false negative results. The positive predictive value of 66% in this study is comparable to the results of Breed et al. (1990), the US collaborative study (Ledbetter et al.; 1992) and Los et al. (1998), which were 64%, 68.4% and 67.8% respectively. The positive predictive value of 75% in the EUCROMIC study (Hahnemann and Vejerslev,1997) is somewhat higher. This is by far the largest study: 62 865 cases. Therefore, the predictive value from the EUCROMIC study might be the most accurate. However, comparison of the accuracy should be done with great caution since the method used and the indication for CVS may differ.

Furthermore in the present study, positive predictive values were calculated for different abnormalities. For trisomy 18 the 100% predictive value in our study is according to results in the literature (Smith et al., 1999). At least 70 cases of trisomy 18 have been confirmed. As far as we aware with the culture method only one discrepancy (Breed, 1992) has been described. Therefore we conclude that trisomy 18, detected in the culture method, should be counselled as a certain abnormal cytogenetic result. For the (semi)direct method, however, several discrepancies (false positive findings) have been described. Hahnemann and Vejerslev (1997b), for

example, found seven cases in the EUCROMIC study. Unfortunately they did not mention the total number of all trisomy 18 cases detected in the study. Therefore it was not possible to calculate the positive predictive value. Breed (1992) gave a predictive value of 80% (95% CI: 52.3- 94.9%) for the direct method. These figures indicate that trisomy 18, detected by the (semi)direct method, is still an uncertain abnormal cytogenetic result.

For 45,X we found at least a 50% positive predictive value. Since one case of possible MCC and one case of a mosaic 45,X/45,XX pattern was not included in the calculation, the predictive value might even be higher. The result of the present study would then correspond with a predictive value of 78% for the culture method by Pittalis et al. (1994). For the direct method 47% was given however.

For the mosaic cases the predictive value is very low in both the (semi)direct and the culture method, although the number of mosaic cases seems to be lower in CVS culture. Therefore in amniocytes in fewer cases a follow-up study is necessary. In the present study in 35 cases (1.8%) a second prenatal test (amniocentesis) was necessary. In the study of Los et al.(1998) this percentage was 2.1% for the (semi)direct method.

Predicted value for indications ‘carrier’ and ‘US abnormalities’ have the expected (nearly) 100%. All other indications have a predicted value of 50- 60%. Since we found no differences in the predictive value for these indications, we do not advise CVS only for women older than 40 years (Los et al., 1998) but for all woman of 36 years and older. Therefore we conclude that positive predictive value depends not on the indication, but on the cytogenetic result found.

A CPM of 1.8% in the present study is consistent with frequencies of CPM of 1 - 2% in other studies (Association of Clinical Cytogeneticists Working Party on Chorionic Villi in Prenatal Diagnosis, 1994; Leschot et al., 1996; Hahnemann and Vejerslev, 1997; Los et al., 1998). We found seven cases with a mosaic structural abnormality in the 35 CPM cases in our study. In these cases we speculate that the aberration might be a culture artefact rather than a real abnormality confined to the placenta. In such situations, especially in cases of balanced aberrations, in

combination with a pregnancy without any anomalies, it is arguable whether a follow-up study in amniocytes should be advised, since the incidence of mosaic structural abnormalities in the population is negligible (Leegte et al., 1998). Moreover, when a mosaic structural abnormality is detected, the risk figure of mental retardation and/ or congenital abnormalities is low. Taking this into consideration, in the present study 1.4% of CPM (28 cases) rather than 1.8% is the correct frequency.

In the present study, the overall frequency of MCC is high (3.6%) in comparison to other studies. In the UK study (Association of Clinical Cytogeneticists Working Party on Chorionic Villi in Prenatal Diagnosis,1994) 0.5% MCC and in the US study (Ledbetter et al.,1992) 2.16% MCC at transcervical sampling was found. Only Smidt-Jensen et al. (1993) found a higher percentage of MCC, namely 4.2%. The reason for the high percentage in the present study could be that all male karyotypes were carefully screened for XX cells. However, the percentage quoted in the collaborative studies are not relevant for cytogenetic diagnosis. More important is the percentage of MCC per patient. Only at a high percentage of MCC or total overgrowth of maternal cells is it possible to miss the correct diagnosis in chorionic villi. In the present study this was not the case. In the UK study, however, total MCC occurred in six individual cases, while an overall percentage of 0.5% MCC was given.

In the present study we found IUD in 17% and IUGR in 5% of the CPM cases. The risk of poor pregnancy outcome might be even higher if the mosaic structural abnormal cases (probably culture artefacts) were excluded. This result confirms the increased risk of poor pregnancy outcome described in the literature. Breed et al.(1991) reported 16.6% IUD and Wapner et al. (1992) 8.6% IUD. However, Goldberg and Wohlferd (1997) were unable to correlate CPM with poor pregnancy outcome. Since the number of CPM cases in the present study is small, the percentage of poor pregnancy outcome is more an indication than a precise risk figure. However, Morssink et al. (1996) measured elevated levels of maternal serum human chorionic gonadotropin (MShCG) in pregnancies with CPM. This resulted in a negative effect on placental function and foetal development.

In conclusion, the reliability of the culture method alone is in the present study comparable to the combination of (semi)direct and culture methods and more reliable than the (semi)direct method alone. The predictive value is acceptable for all indications. The predictive value for trisomy 18 and 45,X is higher in the culture method than in the (semi)direct method alone. MCC should be closely investigated to avoid maternal overgrowth. We therefore conclude that the culture method alone is reliable for prenatal diagnosis and may be used as the sole investigative method.

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Chapter 4.3

Probability tables for exclusion of mosaicism in prenatal diagnosis

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4.3.1 Summary

The decision concerning the number of metaphases that need to be analysed to detect mosaicism of a certain degree depends mainly, for the same confidence levels, on the culture method used (*in situ* or *flask* methods). Several probability tables, designed for either the *in situ* or the *flask* method, have been reported and can be used to assist laboratories in making the decisions referred to above. However, there are instances where part of the analysis is done using the *in situ* and *flask* methods. In such situations, the previously published tables are of limited use. We have generated new table that can be used in such situations, as well as in cases where only the flask method is used.

Keywords:

amniotic fluid culture; chromosomal mosaicism; probability tables; prenatal diagnosis

4.3.2 Introduction

True chromosomal mosaicism is a rare finding in amniotic fluid cultures, but its exclusion represents an obvious major concern for most laboratories involved in prenatal diagnosis (Hsu et al., 1992).

Several tables have been generated to determine the optimum number of metaphases to be counted to exclude (detect) chromosomal mosaicism for either the *in situ* or *flask* culture method (Claussen et al., 1984; Cheung et al.,1990; Rischkind and Risch; 1990; Featherstone et al., 1994). However, for laboratories (like ours) using the in situ culture technique, there are instances where not enough colonies can be analysed (or counted) to exclude mosaicism with the usual confidence. In these situations, a back-up *in situ* culture is usually trypsinized and the respective cells divided over culture flasks, from which chromosomal analysis will proceed further.

Although it is possible to count how many colonies were present in the coverslip prior to trypsinization, after trypsinization it is no longer possible to know which

metaphases come from which colony. For the calculation of the number of cells that need to be analysed from these cultures, the tables referred to above are of limited use.

A similar difficulty can occur when using the flask method, in cases where a small sample of amniotic fluid (hence with fewer cells than usual) is cultured.

We have calculated the number of metaphases that need to be analysed in order to be 95 per cent certain that at least one cell of a desired number of colonies has been observed. The same table can be used to determine the number of metaphases required for accurate analyses using only the flask method.

4.3.3 Mathematical calculation

Assuming that a coverslip contains, prior to trypsinization, N equal-sized colonies of cells, a sample of K cells is analysed after trypsinization and harvesting. The question is: what is the probability that M colonies are represented by at least one cell in the sample? For the exact derivation the reader is referred to the Appendix.

A Pascal program has been written to evaluate the recursive expression. The result is stored as probability distributions for the number of colonies *M* from which cells are observed, conditional on the number *N* of colonies present and the number of cells *K* sampled. Then the number of colonies for which there is 95 per cent certainty that they have been observed, given the number of cells sampled, is computed. This computation is done downwards from the highest sample number present, such that the lowest sample number is obtained for every entry in the table.

4.3.4 Results

Table 1 shows the number of metaphases that need to be analysed to be 95 per cent certain that at least the horizontal number of different colonies have been sampled, when the number of colonies present in the culture is given by the vertical number.

Table 1 – Required number of samples to observe cells from M different colonies out of N colonies present. 95 percent probability table

No. of colonies N present	No. of colonies M needed																												
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
2	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	4	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	4	7	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	3	6	11	21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	3	6	9	14	27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	3	5	8	12	18	33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	3	5	7	11	15	22	38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	3	5	7	10*	13	18	26	44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	3	5	7	9	12	16	22	31	51	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	3	5	7	9	12	15	19	25	35	57	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	3	4	6	9	11	14	18	22	29	39	63	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	3	4	6	8	11	13	16	20	25	32	44	70	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	3	4	6	8	10	13	16	19	23	29	36	48	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	3	4	6	8	10	12	15	18	22	26	32	40	53	83	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	3	4	6	8	10	12	14	17	20	24	29	35	44	58	90	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	3	4	6	8	9	12	14	17	19	23	27	32	39	48	63	0	0	0	0	0	0	0	0	0	0	0	0	0	0
18	3	4	6	7	9	11	14	16	19	22	26	30	35	42	52	67	0	0	0	0	0	0	0	0	0	0	0	0	0
19	3	4	6	7	9	11	13	16	18	21	24	28	33	38	46	56	72	0	0	0	0	0	0	0	0	0	0	0	0
20	3	4	6	7	9	11	13	15	18	20	23	27	31	36	42	49	60	77	0	0	0	0	0	0	0	0	0	0	0
21	2	4	6	7	9	11	13	15	17	20	23	26	29	34	39	45	53	64	82	0	0	0	0	0	0	0	0	0	0
22	2	4	5	7	9	11	12	15	17	19	22	25	28	32	37	42	48	57	68	87	0	0	0	0	0	0	0	0	0
23	2	4	5	7	9	10	12	14	16	19	21	24	27	31	35	39	45	52	60	73	0	0	0	0	0	0	0	0	0
24	2	4	5	7	9	10	12	14	16	18	21	23	26	30	33	37	42	48	55	64	77	0	0	0	0	0	0	0	0
25	2	4	5	7	8	10	12	14	16	18	20	23	26	29	32	36	40	45	51	59	68	81	0	0	0	0	0	0	0
26	2	4	5	7	8	10	12	14	16	18	20	22	25	28	31	35	39	43	48	54	62	72	86	0	0	0	0	0	0
27	2	4	5	7	8	10	12	13	15	17	20	22	24	27	30	33	37	41	46	51	58	66	76	90	0	0	0	0	0
28	2	4	5	7	8	10	12	13	15	17	19	22	24	27	29	32	36	40	44	49	54	61	69	80	0	0	0	0	0
29	2	4	5	7	8	10	11	13	15	17	19	21	24	26	29	32	35	38	42	47	52	57	64	73	84	0	0	0	0
30	2	4	5	7	8	10	11	13	15	17	19	21	23	26	28	31	34	37	41	45	49	55	61	68	77	88	0	0	0

*Example: the analysis of ten metaphases is necessary to be at least 95 percent sure that at least five different colonies have been sampled out of nine equal-sized colonies present.

4.3.5 Discussion

In an attempt to define the minimum number of metaphases which need to be analysed for a reliable prenatal diagnosis, several calculations have been made taking into account the culturing method used (*in situ* or *flask* methods) and the desired confidence levels for exclusion of mosaicism (Claussen et al., 1984; Cheung et al., 1990; Richkind and Risch, 1990; Featherstone et al., 1994).

Some of these calculations resulted in different probability tables, depending on different assumptions concerning the culture method used. However, even for cytogenetic laboratories (like ours) using the *in situ* method, there are instances where not enough colonies can be analysed to allow exclusion of chromosomal mosaicism with the usual confidence. In such situations, a back-up culture is usually trypsinized and the cells are inoculated onto two new coverslips, one of which will be harvested when a sufficient number of mitoses is observed.

Since this trypsinization step obviously disrupts colony integrity, the probability tables used for the *in situ* method are no longer applicable to determine the number of metaphases that still need to be analysed. On the other hand, since part of the analysis has been done from an *in situ* culture, the published tables for the flask method (refs.) are also not directly applicable in such circumstances.

We have therefore generated a new table that can be used to determine quickly the number of metaphases that need to be analysed from a sample of amniotic fluid in order to exclude chromosomal mosaicism with a predefined confidence level (95 per cent). The table is of use for both the flask method alone and the situations referred to above where part of the analysis is done from an *in situ* culture and part from a trypsinized culture.

To illustrate the use of the table, we discuss a few applications. For a laboratory using the *in situ* method and wishing to detect 20 per cent mosaicism with 95 per cent confidence, 14 metaphases, derived from different colonies, need to be analysed irrespective of the total fluid volume (Richkind and Risch, 1990). If, however, only ten colonies could be analysed from the *in situ* culture, four additional colonies must be sampled from the trypsinized culture to achieve a similar sensitivity level of mosaicism

detection. The decision as to how many metaphases need to be analysed from the trypsinized culture will obviously depend on the total number of colonies present prior to trypsinization. This number can be obtained by counting the actual number of colonies in the culture, or by using the estimation of Richkind and Risch (1990), i.e., assuming the formation of 1.5 or 2 colonies per ml of cultured amniotic fluid. Supposing that there were eight colonies prior to trypsinization, seven metaphases would need to be analysed to be 95 per cent confident that at least four different colonies had been sampled.

The total sensitivity will be somewhat lower (90.25 per cent) since the two sensitivities (95 per cent confidence to detect mosaicism and 95 per cent confidence to sample different colonies) need to be multiplied.

Our calculations, like those of Claussen et al. (1984), Richkind and Risch (1990), Cheung et al. (1990), and Featherstone et al. (1994), obviously assume equal colony sizes and contributions to the pool of analysable metaphases in the trypsinized culture. When there are colonies of different size, only medium or large-sized colonies should be counted and used in the calculation since these are more likely to contribute to the pool referred to above.

The probability table (Table 1) presented here can also be used in laboratory setting using only the flask method. Accordingly, if one wants to be 95 per cent confident that a mosaicism ≥ 20 per cent is detected, and if the total amount of fluid collected was 20 ml, a total number of 23 metaphases have to be analysed, assuming the presence of 30 "colony-forming" cells in the cultured sample (Richkind and Risch, 1990). Smaller amounts of amniotic fluid imply, for the same sensitivity and confidence levels, the analysis of more metaphases.

The calculations presented here, and corresponding probability tables, provide simple guidelines that can help laboratories standardise the confidence levels of prenatal diagnosis, either when they rely on the flask method alone or when, despite using the *in situ* method, they need to use trypsinized cultures to complete the analysis.

4.3.6 References

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Appendix

The question is: what is the probability that *M* colonies are represented by at least one cell in the sample?

Solution (assuming an equal contribution of the different colonies)

If one cell is sampled, one colony is present. We represent the probability that *M* colonies are present in a sample of *K* cells, assuming *N* colonies by *P(M,K,N)*. *M* may vary from 1 (at least one colony is represented in the sample) to the lower of the numbers *K* and *N*, denoted min *(K,N)* (there may not be more colonies than there are samples and there may not be more colonies than there are colonies).

The computation of *P(M,K,N)* is recursive. Assume that we want to compute *P(M,K,N).* This probability is related to the probability that in *K*-1 samples, *M* and *M-1* colonies are present:

P(M,K,N)= (N - M + 1)/

N x P(M - 1,K - 1,N) + (M)/

N x P(M,K -1,N) if K≤N,

assuming that P(M,K,N)=0 if M > min(K,N) or M < 1.

The recursion stops at P(1,1,N)=1.0.

The factor (N - M + 1)/N relates to the probability that a cell is observed from the not yet observed pool of cells, while the factor (M)/N is the probability of observing a cell from an already observed pool.