

Reviews

Preimplantation genetic diagnosis of numerical and structural chromosome abnormalities



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Santiago Munné has been director of PGD at Saint Barnabas Medical Center since 1995. His group there focuses on identifying genetically normal embryos. Originally from Barcelona, Spain, Dr Munné gained his PhD in genetics from the University of Pittsburgh and joined Dr Jacques Cohen at Cornell University Medical College, New York in 1991. There he developed the first PGD test to detect embryonic numerical chromosome abnormalities. His work has been recognized by several prizes: in 1994, 1995 and 1998 from the Society for Assisted Reproductive Technology, and in 1996 from the American Society for Reproductive Medicine. Recently his PGD team has shown higher pregnancy rates in women of advanced age undergoing PGD. This team has performed more than 100 PGD cycles for translocations and over 600 PGD cycles for chromosome abnormalities related to advanced maternal age. Dr Munné has more than 100 publications to his name, and is a frequent lecturer, both nationally and internationally, on his team's work and the field of preimplantation genetics.

Abstract

The causes of the decline in implantation rates observed with increasing maternal age are still a matter for debate. Data from oocyte donation strongly suggest that in women of advanced reproductive age, the ability to become pregnant is largely unaffected while oocyte quality is compromised. The incidence of chromosomal abnormalities in embryos is considerably higher than that reported in spontaneous abortions, suggesting that a sizable percentage of chromosomally abnormal embryos are eliminated before any prenatal diagnosis. Such loss may partly account for the decline in implantation in older women. Because of the correlation between aneuploidy and reduced implantation, it has been postulated that selection of chromosomally normal embryos could reverse this trend. Preimplantation genetic diagnosis (PGD) for aneuploidy had three objectives relevant to the present paper: (i) to increase rates of implantation, (ii) to reduce risks of spontaneous abortion, and (iii) to avoid chromosomally abnormal births. Implantation rates did not increase when only five chromosomes were analysed in blastomeres. With eight chromosomes, a significant increase in implantation was achieved. PGD can significantly reduce the incidence of spontaneous abortion. In our clinic, a significant decrease in spontaneous abortions was found, from 23 to 11% after PGD. Currently in cases diagnosed at Saint Barnabas, 0.8% chromosomally abnormal conceptions have been observed after PGD versus an expected 3.2% in a control age-matched group. It seems clear that PGD reduces the possibility of trisomic conceptions under all conditions. If a couple's main interest is to improve their chances of conceiving (improve implantation), then one should consider maternal age and number of available embryos. Improvements in conception after PGD again increase after 37 years of age with eight or nine probes. Carriers of translocations are at a high risk of miscarriage or chromosomally unbalanced offspring, and a high proportion have secondary infertility. PGD of translocations has been approached through a variety of methods, here reviewed, and has resulted in a significant reduction in spontaneous abortions. However, implantation rates in translocation carriers are directly correlated with the proportion of normal gametes, and male patients with 70% or more unbalanced spermatozoa have great difficulty in achieving pregnancy with PGD.

Keywords: implantation rate, numerical chromosome abnormalities, preimplantation genetic diagnosis

PGD of numerical chromosome abnormalities

Introduction

Numerical chromosome abnormalities are the major cause of inherited diseases, with an incidence of 21% in spontaneous abortions (Hassold *et al.*, 1980; Warburton *et al.*, 1980, 1986). Of these, trisomies for gonosomes and chromosomes 21, 18, 16 and 13 account for 50% of chromosomally abnormal abortions. In contrast to single gene defects, numerical

chromosome abnormalities occur *de novo*. The only risk factor known is maternal age, with the detection of trisomy by amniocentesis increasing from 0.6 to 2.2% from age 35 to age 40 years (Hook *et al.*, 1992). Thus, the screening of chromosome aneuploidies in human embryos by fluorescence in-situ hybridization (FISH) using X, Y, 18, 13 and 21 probes should significantly reduce the risk of older IVF patients delivering trisomic offspring. Ploidy assessment of single blastomeres by FISH was first achieved in a time frame compatible with IVF in 1993 (Munné *et al.*, 1993). In that work, it was postulated that preimplantation genetic diagnosis

(PGD) of numerical chromosome abnormalities may increase the pregnancy rate in women of advanced maternal age undergoing IVF.

The causes of the decline in implantation observed with increasing maternal age are still under debate, with some authors proposing that maternal age affects uterine receptivity while others indicate that it mostly affects oocyte viability. Nevertheless, the high implantation obtained using oocyte donation, where some control can be exerted over donor age, and uterine factors could be measured, strongly indicates that the oocyte is the major cause of implantation with advancing maternal age (Navot *et al.*, 1994). Ooplasmic components may be involved (Keefe *et al.*, 1995; Cohen *et al.*, 1998; Barritt *et al.*, 2000), as well as zona pellucida thickening (Cohen 1993; Meldrum *et al.*, 1998), but the clearest link so far between maternal age and embryo competence is aneuploidy. The increase in aneuploidy with maternal age in spontaneous abortions and live offspring was also found in both cleavage-stage embryos (Munné *et al.*, 1995a; Márquez *et al.*, 2000) and unfertilized oocytes (Dailey *et al.*, 1996). The rate of chromosomal abnormalities in embryos was higher than that reported in spontaneous abortions, suggesting that a sizable number of chromosomally abnormal embryos are eliminated before clinical recognition. Such loss of embryos could account for the decline in implantation with maternal age. For instance, the rates of embryonic monosomy and trisomy are similar (Munné *et al.*, 1995a), while with the exception of monosomy 21 (1/1000 karyotyped abortions), the other autosomal monosomies are normally undetected in clinically recognized pregnancies. Furthermore, monosomies in mice (Magnuson *et al.*, 1985) and human (Sandalinas *et al.*, 2001) do not develop to blastocyst stage, with the exception of human monosomy 21 and X. This is in agreement with the observation that blastocyst formation declines with maternal age in women over 30 years old (Janny and Menezo, 1996). It is not known whether trisomies that develop to term (13, 18, 21) have a lower implantation rate than normal embryos. However, even recognized pregnancies with trisomy 21 spontaneously abort in 84–93% of cases depending on the age of the mother (Warburton *et al.*, 1986).

Because of the correlation between aneuploidy and declining implantation rates with maternal age, it was postulated that negative selection of chromosomally abnormal embryos could reverse this trend (Munné *et al.*, 1993). Currently, negative selection of aneuploid embryos can only be done through PGD, either by polar body or blastomere analysis. Low metaphase yield and less than 30% of karyotypable metaphases, together with the requirement of overnight culture in antimitotics (Santaló *et al.*, 1995), make karyotype analysis unsuitable for PGD. FISH allows chromosome enumeration to be performed on interphase cell nuclei, i.e. without the need for culturing cells or preparing metaphase spreads. FISH has been applied to PGD of common aneuploidies using either human blastomeres (cells from 2- to 16-cell stage embryos) or oocyte polar bodies (Munné *et al.*, 1993, 1995a, 1995b, 1998a, 1998b, 1998c; Verlinsky *et al.*, 1995, 1996, 1998a,b; Manor *et al.*, 1996; Munné and Weier 1996; Verlinsky and Kuliev 1996; Gianaroli *et al.*, 1997, 1999a,b). Currently, probes for chromosomes X, Y, 13, 14, 15, 16, 18, 21 and 22 are being used simultaneously (Bahçe *et al.*, 2000), with the potential of detecting 70% of the aneuploidies detected in spontaneous abortions.

FISH technique for the preimplantation diagnosis of aneuploidy

Several FISH protocols for simultaneous detection of multiple chromosomes with specific probes have been proposed and applied. Several types of protocols have been used to maximize the use of a limited number of fluorochromes to study as many chromosomes as possible. One approach was to use ratios of fluorochromes, labelling five or more chromosomes with only three fluorochromes (Nederlof *et al.*, 1990; Dauwerse *et al.*, 1990; Munné *et al.*, 1995a, 1998b). However, the use of mixtures of colours has the disadvantage that overlapping signals from two different chromosomes sharing one or more colours may produce a misdiagnosis. For that reason, new colours have been developed, such as Spectrum Gold and Spectrum Blue (Vysis). The company Vysis has now released probes labelled with five different fluorochromes, which allow the simultaneous analysis of X, Y, 13, 18, and 21 chromosomes in blastomeres or 13, 16, 18, 21, 22 in polar bodies. Still, with this approach, only five chromosomes can be analysed simultaneously.

To solve this problem, once the cells are analysed for a set of chromosomes, they can be re-analysed with a different set of probes as demonstrated previously (Benadiva *et al.*, 1996; Martini *et al.*, 1997; Bahçe *et al.*, 2000). The second set of probes works with high efficiency (>95%) as demonstrated by analysing the same chromosome in both hybridization cycles (Martini *et al.*, 1997; Bahçe *et al.*, 2000). This, coupled with fast protocols either with conventional denaturation and hybridization protocols or with microwave devices (Harper *et al.*, 1994; Drury *et al.*, 1997), allows the analysis of 10 or more chromosomes simultaneously in a single interphase nucleus in a time frame compatible with regular IVF (Munné *et al.*, 1998c; Gianaroli *et al.*, 1999a; Bahçe *et al.*, 2000).

Liu *et al.* (1998) and Vollmer *et al.* (2000) have also published protocols to recycle the same cell three or more times, but the efficiency drops below 80% by the third hybridization.

Scoring criteria of single cells

The method of differentiation between a split target producing two hybridization signals, and two targets close together is as follows. When their distance apart was at least two domains, a domain being the diameter of a signal, it was taken as being two separate signals. Any others were considered split signals. This criterion was applied to PGD using probes for chromosomes X, Y, 13, 18 and 21 with or without chromosome 16. After PGD, 198 embryos that were not replaced were fully biopsied and all cells analysed. The PGD results were confirmed in 91% of these embryos, with 1.1% (1/88) of the embryos being misclassified as normal, and 17% (19/110) of the embryos classified as abnormal being normal (Munné and Weier, 1996; Munné *et al.*, 1998b). More errors were caused by missing, than by extra signals. Compared with previous protocols (Munné *et al.*, 1993), the above criterion minimizes the risk of transferring abnormal embryos after PGD analysis, but a fraction of normal embryos are not being transferred after erroneous abnormal classification.

From these studies, it is also evident that some probes produce more misdiagnoses than others. Use of the 13/21 alpha-

satellite probe has been discontinued because it produced more misdiagnosis than the individual and locus specific-probes for chromosomes 13 and 21 (Munné and Weier, 1996). Other probes, such as those for chromosomes Y and 18, produce more errors than those for chromosomes X, 13, 16 and 21, probably because they are bigger and tend to split more often (Munné *et al.*, 1998b). These probes could be substituted for smaller ones and would presumably produce fewer errors.

Sources of FISH errors and PGD misdiagnosis

A problem shared by all these approaches is that the diagnosis at the preimplantation stage of embryonic development does not take into account the occurrence of chromosome mosaicism. To differentiate between mosaics and technical errors, it is paramount to analyse all the cells of an embryo.

Criteria for FISH failures

A scoring criterion for differentiating false-positives and false-negatives from mosaicism has been previously described (Munné *et al.*, 1994a). This criterion only applies when all or most of the cells of an embryo are analysed.

The specific FISH signals detected in a given blastomere were considered to reflect a true chromosome constitution in the following instances: (i) Blastomeres with two specific signals for gonosomes and two signals for each autosome analysed; these were considered diploid blastomeres. (ii) Embryos in which all the blastomeres had the same abnormality, such as aneuploid, haploid or polyploid embryos. (iii) Individual blastomeres that had only one signal per chromosome pair.

Table 1. Risk of PGD misdiagnosis due to mosaicism. Number of embryos analysed with at least X, Y, 13, 18, 21 probes was 1903.

<i>Risk (%) of classifying an abnormal embryo as normal:</i>	(A) Overall frequency	(B) normal cells	(AxB) risk of misdiagnosis
2N/POL (detrimental)	3.7	34.8	1.3
Chaotic (detrimental)	12.7	9.8	1.2
Mitotic non-disjunction (all ^a)	7.5	24.2	1.8
Total			4.3

<i>Risk (%) of classifying a mostly normal embryo as abnormal:</i>	(A) Overall frequency	(B) normal cells	(AxB) risk of misdiagnosis
2N/POL (benign)	3.9	23.1	0.9
Chaotic (benign)	1.5	24.9	0.4
Total			1.3
Total misdiagnosis rate due to mosaicism			5.6

^aMitotic non-disjunction was considered detrimental regardless of the number of abnormal cells present in the embryo because the abnormal cells may become part of the fetus. Data from Munné *et al.* (2002).

These were considered haploid cells. (iv) Individual blastomeres that had three or more signals per chromosome pair. These were considered polyploid cells. (v) Individual blastomeres that had extra or missing signals that were compensated by extra or missing signals in sibling blastomeres. We considered that these blastomeres belonged to an embryo with mosaicism generated by mitotic non-disjunction. (vi) Blastomeres showing fewer signals than their sibling blastomeres and belonging to mosaic embryos resulting from the uneven cleavage of a blastomere without previous DNA synthesis. An example would be an embryo with mostly XX 1313 1818 2121 cells, plus XO 13O 1818 OO and XO 13O OO 2121 cells. (vii) The same criteria (i to vi) were also used for multinucleated blastomeres. (viii) Blastomeres with more or less than two gonosomes or chromosome 13, 18 or 21 specific signals, were considered respectively to be FISH false-negative or false-positive errors unless one of the prior criteria (A to G) applied.

Mosaics

Mosaicism cannot be detected efficiently by PGD unless all cells lines are abnormal. However, not all mosaics are equal and depending on the type of mosaic the risk of misdiagnosis and the outcome of the misdiagnosis can be different.

Thirty percent of 2000 embryos analysed were mosaics. Of those, about 500 mosaic embryos were fully analysed (Munné and Cohen 1998; Munné *et al.*, in preparation). There were three main types of mosaics: (i) Chaotic mosaics were the most common (49%). In chaotic mosaics, as first described by Delhanty *et al.* (1993), most abnormal cells are chromosomally different from each other as if random chromosome distribution had occurred. Chaotic mosaics had on average 84% chromosomally abnormal cells. (ii) Diploid/polyploid mosaics accounted for 26% of mosaics, with 43% of their cells being abnormal. (iii) Finally, mosaics produced by mitotic non-disjunction or mitotic anaphase lag accounted for 25% of mosaics and formed on average 65% abnormal cells.

Mosaic embryos with more than three out of eight abnormal cells are classified as detrimental mosaics, which probably do not implant. Those with fewer abnormal cells are called benign mosaics. The chances of misdiagnosis produced by mosaics are about 5.6%, as shown in **Table 1**.

What is the probability of these mosaics producing abnormalities at birth? In a recent article by Evsikov and Verlinsky (1998), mosaicism in blastocysts was found only in 10.5% of the embryos, which had an average of five aneuploid cells per blastocyst. Their results indicate a strong selection at morula–blastocyst transition against some, although not all mosaic embryos.

False monosomies produced by overlaps

The occurrence of missing signals may indicate either a monosomic cell or a failure of the technique to display the remaining signals. Causes of reduced hybridization efficiency have been attributed to loss of DNA during denaturation or fixation, poor probe penetration, insufficient binding of detection reagents or overlap of chromosome-specific signals when multiple probes are used (West *et al.*, 1987; Handyside

1993). It has been found that poor spread of the nucleus during fixation and content of DNA per nuclei increased signal overlap (Munné *et al.*, 1996). When two signals from the same chromosome overlap a single signal is observed, therefore producing a misdiagnosis. The more the nucleus is spread during fixation, the less overlapping of signals and missing signals were found.

The Carnoid method (Tarkowski, 1966), as slightly modified by our team (Munné *et al.*, 1996), produced on average blastomere nuclei of 69 microns under appropriate humidity and temperature conditions. Other fixation methods, such as with Tween 20/HCl (Harper *et al.*, 1994), produce significantly smaller nuclei and may result in more signal overlaps. A new fixation method, which is a combination of the previous two, has been recently described (Dozortsev and McGinnis, 2001) but the nuclear diameters obtained with it have not been yet compared with our method.

Another way in which misdiagnosis may occur is by overlaps of different chromosomes labelled with mixtures that share one colour. For example a chromosome labelled in orange, could overlap with another labelled in orange and aqua, with the result that the first chromosome is masked by the second. The use of a protocol using a single colour per chromosome analysed (Bağçe *et al.*, 2000) resulted in a significant reduction in the error rate of false normal and abnormal PGD results, from 4 to 8% when compared with the previous protocols in which similar probes were used (Munné *et al.*, 1998b, 1998c).

False monosomies: loss of micronuclei during fixation

The FISH error is higher in multinucleated blastomeres (MNB) (11.5%, 13/113) than in those that are mononucleate (3.1%, 13/415) (Munné *et al.*, 1994a,b). This is probably due to the fact that many MNB very often contain micronuclei, and during fixation, some of them can get lost more easily than full nucleus producing false negative FISH errors.

We found strong correlation between types of fixation and loss of chromosomes (Munné *et al.*, 1998b). During fixation with acid: acetic fixative, the drops added before the cell breaks allow the cytoplasm to expand, the more drops the more expansion, while the drop post-lysis removes cytoplasm debris, and probably some anuclear DNA. It is therefore possible that the loss of DNA is higher after adding a drop post-lysis when the cell is more expanded (2 drops pre-lysis instead of one) as was observed in this study. The current recommendation is two drops pre-lysis followed by no drops post-lysis, which with appropriate humidity conditions allow a good spreading, few cytoplasm debris, and minimal loss of DNA.

Because one of the most frequent errors in FISH is the occurrence of false monosomies, either produced by overlaps or loss of micronuclei, the transfer of biopsied embryos on day 4 (Grifo *et al.*, 1998; Gianaroli *et al.*, 1999b) has the advantage of allowing an extra day for the rebiopsy of another cell from embryos with monosomies, or no results. For instance in a large series of PGD cases, some embryos were rescued in this way (Gianaroli *et al.*, 1999b).

Multinucleated blastomeres

MNB have been described in both morphologically normal and abnormal human embryos and they occur in about one-third of the embryos (Tesarik *et al.*, 1987; Winston *et al.*, 1991; Hardy *et al.*, 1993). We have shown previously that MNB are not suitable for the preimplantation diagnosis of aneuploidy because the number of chromosomes in each nucleus varies greatly (Munné and Cohen, 1993). However, when the MNB is binucleate, and both nuclei are chromosomally normal, the remainder of the embryo is also normal (Munné and Cohen, 1993).

False positives: split signals and chromatids

False positive results may occur when a signal is split because of excessive stretching of the DNA during fixation. This occurs more often with some probes than with others. For instance, in a study evaluating split signals for chromosomes X, Y, 13, 18 and 21, the probe for chromosome 13 split the most (Munné and Weier, 1996). Another source of false positives could be the occurrence of an S-phase and non-synchronous replication timing, with one chromosome showing a single signal for its sole chromatid and the other sending two close signals, one for each chromatid (Mukherjee *et al.*, 1992).

The distance between two hybridization signals, specific for the same chromosome, were measured in domains, with a domain being the diameter of one of these signals. Therefore, each domain for each chromosome type had a different area. Based on the reanalysis of all the cells of a group of embryos, it was possible to differentiate between false positives, and mosaicism. In the cases in which two signals were close enough to either be a split signal or two homologue chromosomes in tight proximity, our criteria of scoring them as two separate chromosomes when the signals were two or more domains apart, produced fewer misdiagnoses (Munné and Weier, 1996).

This criterion is not valid for first polar bodies where artefactual (Dailey *et al.*, 1996) and genuine (Angell *et al.*, 1997) chromatid predivision is widespread, and the chromatids are usually found many domains apart.

PGD misdiagnosis

The most reliable way of assessing PGD misdiagnosis is by reanalysing the non-transferred embryos. Our latest data indicate a 7.2% misdiagnosis rate, of which 5.6% is attributable to mosaicism (Munné *et al.*, in submission) as shown in **Table 2**.

Polar body analysis

Preconception diagnosis was pioneered by Verlinsky and coworkers (Verlinsky *et al.*, 1990) for single gene defects. This approach comprises analysing the first polar body alone or in combination with the second polar body in order to determine the genetic status of the oocyte. Since the first polar body is a mirror image of the egg, the occurrence of an extra univalent chromosome (a chromosome with two chromatids) in the first polar body would imply that the egg is nullisomic and that the resulting embryo is monosomic for that particular

Table 2. PGD misdiagnosis ascertained through reanalysis. Source: Munné *et al.* (2002).

PGD diagnosis	Re-analysis	No. embryos
Normal	Normal	359
	Mosaic detrimental	8 ^a
	Aneuploid	5 ^a
Aneuploid	Aneuploid	212
	Mosaic detrimental	50
	Normal or mosaic benign	31 ^a
Other abnormal ^b	Mosaic detrimental, polyploid or haploid	166
	Aneuploid	34
Total	Normal or mosaic benign	20 ^a
		885
Misdiagnosed (%)		64 (7.2)

^aPGD misdiagnosis.

^bThese being polyploid, haploid or complex abnormal (three or more chromosomes, but not all chromosomes being non-disomic).

chromosome. Similarly, the lack of a full univalent in the first polar body would imply the resulting embryo would be trisomic for that chromosome.

Alternatively, premature separation of chromatids (predivision) may occur (Angell, 1991). When predivision occurs at meiosis-I, aneuploid MII oocytes with 23 univalents plus one chromatid, and those with 22 univalents plus one chromatid, may recover the normal chromosome constitution (23 univalents) during the second meiotic division if the extra or missing chromatid is favourably distributed into the oocyte and second polar body. Consequently, the second polar body should be also analysed to prevent a misdiagnosis.

The importance of predivision as a contributing mechanism to overall aneuploidy is still under debate. Most studies after Angell (1991), and their description of predivision have been able to detect it. Its frequency varies greatly depending on the study (Dailey *et al.*, 1996; Angell 1997; Boiso *et al.*, 1997; Marquez *et al.*, 1998; Verlinsky *et al.*, 1998a, 1998b, 1999; Mahmood *et al.*, 2000; Sandalinas *et al.*, 2002).

Advantages

Polar body analysis has the advantage that is more acceptable for couples who do not approve of discarding chromosomally abnormal embryos. For instance, even zygotes can be frozen while the second polar body is being tested, and then permitting only those that are normal to proceed to syngamy. In addition, the first and second polar bodies are not involved in embryo development. Their removal does not decrease rates of fertilization, cleavage and blastocyst formation (Verlinsky and Kuliev, 1993).

FISH analysis of first polar bodies, sometimes in combination with second polar body analysis, was first attempted by Verlinsky and coworkers (Verlinsky *et al.*, 1995) and ourselves (Munné *et al.*, 1995b). Since autosomal aneuploidy occurs predominantly in maternal meiosis I (Hassold *et al.*, 1987, 1991; Antonorakis *et al.*, 1991), aneuploidy analysis of the first polar body could detect the great majority of the autosomal aneuploidy identified in blastomeres.

Table 3. Expected and detected trisomies after PGD of aneuploidy. Data up to September 2001.

Expected trisomies				
Age (years)	Conceptions (n) after PGD ^a		Expected trisomies ^b (%)	
30–34	73	×	1.2	= 0.88
35–39	108	×	1.3	= 1.40
40–45	60	×	7.3	= 4.38
Total (%)	241		6.66/241 (2.8)	= 6.66
Detected trisomies after PGD				
One trisomy out of 219 PGD conceptions (%)			1/241 (0.4)	

^aExcluded: (1) spontaneous abortions without karyotype, (2) Rb translocations with chromosome 21 analysed in second panel.

^bEiben *et al.* (1994).

Disadvantages

Using FISH, univalent chromosomes appear as double-dotted signals, with one dot per chromatid. However, the proximity of the chromatids sometimes makes the two dots overlap, so they appear as a single dot. This would not be a problem if predivision was non-existent, but misdiagnoses may occur if it is a common event.

We have detected an extra problem. It seems that predivision increases artefactually in both first polar bodies and eggs with increasing time in culture (Munné *et al.*, 1995b; Dailey *et al.*, 1996). This increase is already apparent 6 h after egg retrieval.

Other possible causes of PB misdiagnosis are the artefactual loss of chromosomes during fixation or the lack of probe penetration in some forms of chromatin. For instance, an excess of missing chromatids in the PB, resulting in an excess of 23 + 1/2 oocyte diagnoses, is probably caused by an error produced either by hybridization error or chromosome loss (Verlinsky *et al.*, 1996, 1999; Rosenbusch *et al.*, 2002).

Another disadvantage of preconception diagnosis of aneuploidy is that paternal inherited aneuploidies, polyploidy, haploidy, and some mosaics cannot be detected. Those can, however, be assessed by analysing blastomeres instead of polar bodies. On the other hand, polar body analysis is not affected by errors produced by mosaicism.

Results of segregation

In 800 cycles of PGD, approximately 5000 oocytes had their first and second PB biopsied (Verlinsky *et al.*, 1998a, 1998b, 1999; reviewed by Verlinsky and Kuliev, 2001). Among these, 82% produced results for at least one PB, and 71% for both. Of the eggs analysed, 42.9% were abnormal oocytes, of which 48.3% had first polar body errors, 29.3% had second polar body errors and 22.4% had errors in both polar bodies. Of the

eggs with errors in both polar bodies, 37.6% involved the same chromosome, thus resulting in a balanced result. The most common abnormality in the first polar body involved chromatids: 51.9% of abnormalities were missing chromatids, 16.6% extra chromatids, 8% missing chromosomes, 0.6% extra chromosomes, and 21.9% complex abnormalities. However, the excess of missing chromatids in the PB resulting in an excess of 23 + 1/2 diagnoses is probably caused by an error produced either by hybridization error or chromosome loss (Rosenbusch *et al.*, 2002). In the second polar body, 40.6% of abnormalities included missing chromatids, 45.8% extra chromatids and the rest were complex abnormalities.

Chromosomes 13, 18 and 21 predominantly displayed first meiosis abnormalities (48.4, 59.8, 46.3%), followed by second meiotic abnormalities (about 20–30%) and both types of abnormalities (the remainder). This information contradicts data derived from postnatal cases in which trisomy 13 and 21 arose in meiosis I in >80% of cases, while trisomy 18 originates in meiosis II in more than half of the cases (Antonarakis *et al.*, 1991; Sherman *et al.*, 1994; Fisher *et al.*, 1995; Lamb *et al.*, 1996; Robinson *et al.*, 1996; Bugge *et al.*, 1998). Results from spectral karyotyping also suggest that most abnormalities in oocytes and polar bodies are caused by predivision of chromatids (Sandalinas *et al.*, 2002).

Karyotyping of polar bodies

First polar body chromosomes are still in metaphase up to 6 h after retrieval, and if well fixed, they can be karyotyped (Márquez *et al.*, 1998). However, because it is difficult to obtain a good spread, fewer than 25% of polar bodies can be analysed with this technique.

Recently, Verlinsky and Evsikov (1999) have developed a method to produce banding-quality metaphases from second polar bodies. The best method to obtain chromosomes from human second polar bodies was to inject the second polar body into enucleated MII oocytes, using the intracytoplasmic sperm injection (ICSI) procedure, although with a larger needle. The oocyte was then activated in order to produce a pronuclei from the nucleus of the second polar body nucleus. The zygote was subsequently cultured for 1 h in okadaic acid and fixed, a method that is 100% effective in producing metaphase chromosomes.

Results of PGD for aneuploidy: trisomic offspring, spontaneous abortions and implantation rates

Reduction in trisomic offspring

So far, more than 2000 cases of PGD of aneuploidy have been performed, either using embryo biopsy or polar body biopsy (Gianaroli *et al.*, 1999a; Munné *et al.*, 1999; Verlinsky and Kuliev 2001). Large numbers are needed to demonstrate a decrease in trisomic offspring, from the 2.6% trisomies for chromosomes 13, 18 or 21 detected in CVS in women 39 years old, to 0.3% after PGD (assuming a 10% error rate). At least 300 fetuses or babies must be conceived by this technique to detect a significant reduction in trisomic offspring.

Indeed, misdiagnoses have already occurred after PGD (Munné *et al.*, 1998c). A reanalysis of the misdiagnosed cells concluded that the errors were due to mosaicism or loss of DNA.

Nevertheless, a rate of 0.4% of 241 fetuses with trisomy 21 has been found (Munné, unpublished) compared with a 2.8% rate expected in a population of the same age range (Eiben *et al.*, 1994) (Table 3).

Decrease in spontaneous abortions

A multicentre IVF study was designed to compare controls with a test group undergoing embryo biopsy and preimplantation genetic diagnosis for aneuploidy. Patients were matched retrospectively, but blindly, for average maternal age, number of previous IVF cycles, duration of stimulation, oestradiol concentrations on day +1, and average mature follicles. All these parameters were similar in test and control groups. Only embryos classified as normal for those chromosomes were transferred after PGD (Munné *et al.*, 1999). The results revealed a similar rate of fetal heartbeats/embryo transferred in control and test groups, even if slightly higher in the test group. However, spontaneous abortions decreased after PGD measured as the ratio of fetuses with heartbeats that aborted against the total number of fetal heartbeats detected, decreased after PGD ($P < 0.05$). Ongoing

Table 4. Chromosome specific aneuploidy rates in human cleavage-stage embryos.

Chromosome	No. analysed embryos	Aneuploid (%) ^a
22	302	17 (5.6)
16	520	27 (5.2)
15	302	15 (5.0)
1	190	9 (4.7)
21	882	38 (4.3)
7	215	7 (3.2)
17	190	5 (2.6)
13	882	21 (2.4)
4	211	4 (1.9)
18	999	17 (1.7)
XY	999	12 (1.2)
6	190	2 (1.1)
14	277	3 (1.1)

^aDouble aneuploidies counted twice, once for each chromosome.

Table 5. Implantation rates by age in 163 PGD cycles and 163 matched controls.

Age (years)	Implantation (FHB/embryos replaced)	
	Controls % (n)	PGD % (n)
35.0–39.0	21.6 (245)	24.7 (162)
39.1–45.0	10.7 (346) ^a	17.8 (196) ^b
Total >35.0	15.2 (591)	20.9 (358)

FHB = fetal heart beat.
a versus b, $P < 0.05$.

pregnancies and delivered babies increased in the PGD group of patients ($P < 0.05$) (Munné *et al.*, 1999).

Increase in implantation rates

Using probes for the same chromosomes used in the first study (Munné *et al.*, 1999), plus probes for chromosomes 15, 16 and 22, a significant two-fold increase was identified in implantation rate (Gianaroli *et al.*, 1999a). The low increase in implantation rates after embryo biopsy and PGD of aneuploidy in the first study (Munné *et al.*, 1999) could be attributed to the probes employed. The chromosomes chosen for that study produce abnormalities compatible with further development or have a low impact on embryo implantation. For instance, after analysing more than 1000 embryos for different chromosomes (Table 4), the most common aneuploidies are those for chromosomes 22, 16, 15, 1 and 21, while those for chromosomes XY and 18 were four times less common (reviewed by Munné *et al.*, 2001, and unpublished data). Thus, when a few more studies were added involving chromosomes 16, 22 and 15 to the original protocol using XY, 13, 18, 21 probes (Munné *et al.*, 1999), the improvement was significant (Gianaroli *et al.*, 1999a).

Reduction in multiple gestations

PGD of aneuploidy may also help reduce the number of multiple pregnancies. In the two latest studies involving a test and a control group, significantly fewer embryos were transferred in the PGD groups than in control groups (Munné *et al.*, 1999; Gianaroli *et al.*, 1999).

Indications for PGD of aneuploidy

Appropriate maternal age

Although prenatal diagnosis is recommended for women aged 35 years and older, PGD may be recommended for younger or older women depending on the benefits observed from past procedures. There is still not enough data to determine when a significant reduction occurs in trisomic offspring. But as shown in Table 5, enough data is beginning to accumulate to indicate that the increase in implantation rate does not occur in 35–37 year old women but mostly in those aged 38 and older (Munné, unpublished).

Recurrent spontaneous abortions

Recurrent miscarriage (RM) has been defined as three or more consecutive spontaneous abortions of less than 20 weeks gestation, including only patients with normal somatic karyotype (Stephenson, 1996). The IVI group of Valencia (Simon *et al.*, 1998; Vidal *et al.*, 1998; Pellicer *et al.*, 1999) have detected significantly more chromosome abnormalities in embryos from women with recurrent miscarriages than in their respective control groups. However, applying PGD to embryos of those patients did not improve implantation rates nor reduce spontaneous abortions.

We performed PGD of aneuploidy in 23 patients (average age 36.9) with recurrent miscarriage (average 3.8) and found that PGD improved the prognosis of these patients. The 13% of losses compared with the expected 37% ($P < 0.016$) predicted

by Brighten *et al.* (1999) on a population with similar previous losses and maternal age (Munné *et al.*, unpublished).

Repeated IVF failure (RIF)

In 27 cycles that had failed three or more IVF cycles it was found that for several patients, all their embryos were chromosomally abnormal, and remaining patients had embryos with high percentages of chromosome abnormalities (54%, $n = 74$ embryos) for an average maternal age of only 32 years. However, some patients then utilized PGD for chromosome abnormalities, and their implantation rates were compared with a control group of similar ages and number of cycles of IVF. The PGD group showed a 17% implantation rate, not statistically higher than the 10% found in the control group (Gianaroli *et al.*, 1999a).

Another group has recently published a series of 23 patients with repeated IVF failure (average maternal age 30 years) that undertook PGD for aneuploidy testing, by scoring chromosomes XY, 13, 18, and 21 (Kahraman *et al.*, 2000). Their 30% pregnancy rate was not compared with a control group.

Towards a full chromosome count

FISH with the currently limited number of fluorochromes cannot alone analyse all chromosomes in a single cell. Several techniques have been tested to produce a full chromosome count. One approach has been to obtain metaphase chromosomes from polar bodies or blastomeres either obtained after biopsy or after converting the cell to metaphase stage (Verlinsky and Evsikov, 1999; Willadsen *et al.*, 1999) and analysing them by spectral karyotyping imaging (SKY) (Márquez *et al.*, 1998) or conventional karyotype analysis. Unfortunately, metaphase chromosomes need to be very well spread to produce an accurate SKY analysis and only a few cases have been reported using SKY (Willadsen *et al.*, 1999).

The other approach has been to use molecular techniques to amplify the whole genome and then do a quantitative analysis either by comparative genome hybridization (Kallioniemi *et al.*, 1992; Wells *et al.*, 1999) or quantitative fluorescence multiplex PCR (QF-PCR) (Mansfield, 1993; Sherlock *et al.*, 1998). Of these methods, SKI, comparative genome hybridization and cell conversion have been applied clinically but only on a handful of cases.

Cell conversion to undergo metaphase

Recently, Verlinsky and Evsikov (1999) have developed a method to produce banding-quality metaphases from second polar bodies. Electrofusion of polar bodies with enucleated mice oocytes reconstitutes the oocyte and can contribute to development or provide metaphases (Verlinsky *et al.*, 1994). The best method to obtain chromosomes from human second polar bodies was to inject the second polar body into enucleated MII oocytes, in the same way as the ICSI procedure, although with a larger needle. The oocyte is then activated in order to produce a pronucleus from the second polar body nucleus. The zygote is subsequently cultured for 1 h in okadaic acid and fixed, which is 100% effective in producing metaphase chromosomes.

A method to obtain metaphase stage chromosomes from blastomeres has been recently published by our team (Willadsen *et al.*, 1999). It is based on the observation that nuclear transfer to freshly matured oocytes (collected 2–6 h after completion of the first meiotic division) results in the transferred nucleus being arrested in a configuration resembling M-II (Willadsen, 1992). Human blastomeres were fused with enucleated cow oocytes as recipients, resulting in the formation of human metaphase-stage nuclei. The addition of colcemid prevented the progression of these metaphases into pronuclear stage. The fused blastomeres could be banded, and analysed with painting probes, or by spectral karyotyping. A baby has been born after using this procedure for the PGD of translocations. An alternative to the Willadsen *et al.* (1999) method is to fuse human blastomeres to mouse zygotes (Verlinsky and Evsikov, 1999). The blastomere nucleus then enters mitosis. The heterokaryons should be cultured in vimblastine, otherwise they will progress into mitosis and produce two cells. Using this approach, an 84% yield of metaphases was obtained when applied method to 19 PGD for translocations (Evsikov *et al.*, 2000). Neither method has yet been applied for the PGD of aneuploidy.

Spectral imaging (SKY)

An alternative to conventional FISH is the use of 24 painting probes, one for each chromosome type, labelled in ratios of five different fluorochromes and observed with spectral imaging. The system measures all points simultaneously in the sample emission spectra, across the visible and near-infrared spectral range. Instead of measuring a single intensity, as in conventional epifluorescence microscopy, the spectral imaging system measures the whole spectrum of emitted light allowing overlapping multiple fluorophores to be differentiated. Artificial colours are then assigned to each chromosome to provide the karyotype. Displayed colours allow all chromosomes to be readily visualized after spectral imaging. Spectra-classification of colours is a chromosome classification algorithm based in spectral measurements at each pixel (Schröck *et al.*, 1996). This technique has recently been applied to oocytes, first polar body and blastomere metaphases (Márquez *et al.*, 1998). Since first polar bodies are found at metaphase stage shortly after retrieval (Munné *et al.*, 1998d), they could be analysed by spectral imaging for purposes of PGD. Similarly, metaphases obtained from blastocyst biopsies have been analysed by SKY (Sandalinas *et al.*, 2001), and blastomeres converted to metaphase stages by methods described below could also be analysed by SKY.

Comparative genome hybridization

Comparative genomic hybridization (Kallioniemi *et al.*, 1992) can accurately determine total or partial aneusomy by loss or gains of DNA, using a combination of PCR and FISH technology. The technique involves the following steps: (1) DNA of the cells to be tested is labelled, for example in green (with FITC-biotin); (2) DNA from cells with normal chromosome complements are used as genomic control DNA, which is labelled with a different colour, for example red (TRITC-Digoxigenin); (3) test and control DNAs are mixed in a 1:1 ratio; (4) the mixture is used as a probe for chromosomal in-situ suppression hybridization, also known as chromosome painting (Pinkel *et al.*, 1988), on metaphase spreads with

normal chromosome complements of known sex; (5) using an image analysis system, the resulting ratio of green/red fluorescence intensities for each chromosome should reflect the number of homologous chromosomes present in the test DNA: 0 for nullisomies, 0.5 for monosomies, 1 for normal cells, 1.5 for trisomies, etc. Similarly, partial monosomies and trisomies will also be detected in the same fashion.

This technique can be used for single cell analysis provided that the whole genome of the cell is previously amplified (Wells *et al.*, 1999). Wells *et al.* (1999) tested four amplification methods and found that DOP-PCR amplified most of the genome (91%) without amplification bias. It produced a much greater quantity of DNA, and allowed labelling by incorporation of fluorescent nucleotides during the second amplification reaction, which yields brighter signals than those obtained by nick translation. In contrast other amplification techniques such as PEP, T-PCR and alu-PCR were inferior (Wells *et al.*, 1999). This technique has been applied to the study of human blastomeres from discarded embryos, and seems to be reliable (Wells and Delhanty, 2000).

Another group (Voullaire *et al.*, 2000) applied a similar method to blastomeres from embryos that were frozen after biopsy, in order to gain enough time for analysis. However, embryo freezing decreases implantation rates, defeating the initial purpose of increasing embryo implantation. Just recently, this technique has been applied to polar bodies obtaining results prior to embryo transfer and without the need of embryo freezing (Wells *et al.*, unpublished).

PGD of structural abnormalities

Balanced translocations occur in 0.2% of the neonatal population, but at a higher rate among infertile couples and patients with recurrent abortions. In a recent report, balanced translocations were found in 0.6% of infertile couples, 3.2% of couples that failed over 10 IVF cycles, and 9.2% among fertile couples experiencing three or more consecutive first-trimester abortions (Stern *et al.*, 1999). It was also found in 2–3.2% of males requiring ICSI (Testart *et al.*, 1996; Meschede *et al.*, 1998; Van der Ven *et al.*, 1998).

PGD can be offered to carriers of balanced translocations as an alternative to prenatal diagnosis and pregnancy termination of unbalanced fetuses. In recent years, PGD for structural chromosome abnormalities has been attempted by a variety of approaches. The aim of PGD for translocations is to reduce the rate of spontaneous abortions and to minimize the risk of conceiving an unbalanced baby.

Approaches to PGD of translocations

Preimplantation genetic diagnosis (PGD) of translocations has been attempted only using FISH, using a variety of approaches.

Metaphase analysis

First polar bodies: This method was proposed after the observation that >90% of first polar bodies fixed for 6 h after retrieval are in metaphase stages (Durban *et al.*, 1998; Munné

et al., 1998d). The translocation can then be identified using chromosome-painting probes for the two chromosomes involved in the translocation (Munné *et al.*, 1998d). This method was later improved by using telomeric probes to enhance the regions not covered by the painting probes. It was also applied to centromere or marker probes, desirably in a third colour (blue), to distinguish chromatids and avoid the confusion between single chromatids and whole chromosomes (Munné *et al.*, 1998e). Single chromatids have been found to occur frequently in degenerating polar bodies (Munné *et al.*, 1995b).

In addition, spectral imaging has been used to identify all 23 chromosomes in polar bodies (Márquez *et al.*, 1998) but this technique requires well-spread chromosomes in order to identify each one of them. This is quite difficult to do on a regular basis with such a small and degenerating cell. So far, spectral karyotyping has not been used clinically to analyse polar body chromosomes.

One problem with this technique is the occurrence of crossing-over and predivision of chromatids. In both cases the outcome of the second meiotic division is unclear, and the second polar body or blastomeres should be analysed. A second problem is the occurrence of an interstitial crossover with subsequent segregation of balanced and unbalanced sets of chromosomes during the second meiotic division. So far, two of these events have been detected (Munné *et al.*, 1998f).

Finally, a third problem concerns the shortness of polar-body chromosomes. This implies that terminal translocations are difficult or impossible to see with painting probes. These probes have then to be reinforced by adding telomere probes to the mixture.

Chromosomes from single blastomeres or second polar bodies by oocyte fusion: Methods to obtain metaphase stage chromosomes from blastomeres have been recently published by two teams. They are based on the fusion of blastomeres to cow eggs or mice zygotes, as described previously in the section of PGD for aneuploidy (Willadsen *et al.*, 1999; Verlinsky and Evsikov, 1999).

The Willadsen approach has been used for two clinical cases of translocation resulting in chromosomally normal offspring (Willadsen *et al.*, 1999). The second approach (Verlinsky and Evsikov, 1999) has also been used clinically in 19 patients (Evsikov *et al.*, 2000).

A similar variant is to inject second polar bodies into oocytes. Unlike the first polar body, the second polar body nucleus is in interphase because it inherits the oocyte cytoplasm possessing chromosome-decondensing activity (Howlett and Bolton, 1985). By injecting it into enucleated MII oocytes, followed by oocyte activation, Verlinsky and Evsikov (1999) were able to produce a pronucleus from the second polar body nucleus. The zygote was then cultured for 1 h in okadaic acid and fixed to produce samples of metaphase chromosomes.

Interphase FISH on blastomeres

FISH on interphase blastomeres can be applied for translocations of any parental origin or for other structural

abnormalities such as inversions. One approach has been to develop specific probes expanding the breakpoints of each translocation (Munné *et al.*, 1998d; Weier *et al.*, 1999) or inversion (Cassel *et al.*, 1997). Another approach is to use probes distal to the breakpoints or telomeric probes in combination with proximal or centromeric probes, either for translocations (Munné *et al.*, 1998g, 2000a; Pierce *et al.*, 1998) or inversions (Iwarsson *et al.*, 1998a). The exception is Robertsonian translocation (RT), for which chromosome enumerator probes are used to detect aneuploid embryos (Conn *et al.*, 1998; Munné *et al.*, 1998g). Only the first approach (spanning probes) can differentiate between balanced and normal embryos. If sufficient normal embryos are available, balanced embryos should not be transferred in order to avoid the perpetuation of the genetic disease in the family.

Breakpoint spanning probes: Breakpoint spanning probes used in interphase nuclei can detect normal, balanced or unbalanced karyotypes resulting from any translocation, inversion, deletion or duplication. They work as follows. When two breakpoint spanning probes, one for chromosome A labelled in red and one for B labelled in green are used, for instance in a translocation case, two independent green and two independent red signals are observed in normal cells. In balanced cells, the normal A appears as an independent red signal, the normal B as an independent green signal and the derivative A and B chromosomes appear as associations of smaller red and green signals. Any other combinations represent unbalanced nuclei. This situation arises because each hybridization target split into two physically separated domains of about equal intensity when the translocation occurred. Therefore, a derivative chromosome appears as an association of a green and a red domain. To further distinguish the derivative chromosomes, a blue fluorescent-satellite probe was added for the centromeric region of one of the chromosomes involved in the translocation.

This approach was first presented for PGD of inversions (Cassel *et al.*, 1997) and later applied to translocation PGD cases (Munné *et al.*, 1998g) and deletions (Iwarson *et al.*, 1998b). Methods used to produce these probes have been described by Fung *et al.* (1998). Breakpoint spanning probes are seldom used because probe development has to be performed for each breakpoint of each translocation and the method is time consuming and expensive.

Telomere probes or probes distal to the breakpoints for translocations: Several groups have used probes distal to breakpoints (Munné *et al.*, 1998g; Pierce *et al.*, 1998; Van Assche *et al.*, 1999) or telomeric probes (Munné *et al.*, 2000). However, in order to identify any possible unbalanced event, two probes distal to the breakpoint and a proximal one, or two proximal and two distal ones should at least be used. The use of only one distal and one proximal probe (Pierce *et al.*, 1998) cannot detect for instance 1:3 unbalanced embryos.

This approach is the most simple of those so far described, thanks to the recent commercialization of telomeric probes for most q and p arms. However, this approach cannot differentiate between normal and balanced embryos. In a series of five cases (Munné *et al.*, 2000), the FISH error rate based on the reanalysis of embryos deemed abnormal after PGD, was between 6 and 10%, which is comparable with other PGD tests

(Munné *et al.*, 1995a; Munné and Weier, 1996).

A more robust design is to use two proximal to the breakpoint and two distal to the breakpoint probes, thus differentiating between unbalanced events and 'non-sense' events produced by FISH errors (Munné *et al.*, 1998g). In contrast, if only three probes are used and a FISH error affects one of the probes, the cell can be misdiagnosed. This has already occurred in our laboratory in one instance when an unbalanced embryo was misdiagnosed as normal based on the result of three probes. This embryo was transferred, implanted and resulted in a chromosomally unbalanced infant (Munné, unpublished). For this reason, the use of four probes, that is two distal and two proximal probes, is strongly recommended.

Pericentric inversions: Pericentric inversions are among the most frequent chromosomal rearrangements in humans, with a frequency of 1–2% of the population (Nielsen and Psilocin, 1975). The risk of unbalanced progeny is caused by the occurrence of an odd number of meiotic crossovers between a normal chromatid and an inverted chromatid, or in rare cases also by U-loop recombination. The risk of unbalanced progeny for inversion carriers occurring through a recombinant is estimated at 5% for males and 10% for females (Sutherland *et al.*, 1976).

Several approaches have been used, of which two can be usefully applied to PGD of inversions. One has been the use of breakpoint spanning probes specific for each translocation, which permit the differentiation of normal, balanced and unbalanced embryos (Cassel *et al.*, 1997). This method is very expensive and time consuming. A better alternative is the use of probes distal to the breakpoints or telomeric probes (Iwarson *et al.*, 1998a), preferably in combination with centromeric or proximal probes to detect whichever recombination type (X or U) has occurred in the inverted region (Escudero *et al.*, 2001).

Paracentric inversions: These unbalanced chromosomes are produced by crossing-over resulting in either acentric or dicentric chromosomes. As with pericentric inversions, a centromeric and a telomeric probe will suffice to detect these imbalances. However, 4% of the offspring of 446 paracentric inversions studied by Pettenati *et al.* (1995) were the product of 'U-loop recombination', which leads to either a duplication or deletion of part of the inverted segment. It is difficult to find probes for these cases because they cannot be detected with telomeric probes, since it is not possible to predict where recombination will occur.

Chromosome enumeration for Robertsonian translocations: Robertsonian translocations arise through the p-arm fusion of acrocentric chromosomes. Hence by using any probes labelling the chromosomes involved, aneuploid embryos can be differentiated from normal or balanced embryos. These are the easiest translocations to analyse by simply using enumerator probes (Conn *et al.*, 1998; Munné *et al.*, 1998g) and many PGD cases have already been performed (Munné *et al.*, 2000).

Uniparental disomy (UPD) has been described for all acrocentric chromosomes. However, only UPD14 and UPD15 have phenotypic consequences, since they have imprinted

regions. The imprinted region of chromosome 15 is 15q11-q13 and UPD15 causes either Angelman or Prader-Willi syndromes (Kuwano *et al.*, 1992). The imprinted region of chromosome 14 is not yet defined and UPD14 produces precocious puberty (Tomkins *et al.*, 1996). Although UPD is rare, when it involves acrocentric chromosomes it is mostly generated from Robertsonian translocation carriers (Tomkins *et al.*, 1996).

Most cases of maternal UPD14 consisted of 45,t(13;14)(q10;q10) with heterodisomy, or 45,t(14;14)(q10;q10) with isodisomy. The mechanism could arise *de novo* through isodisomy 45,t(14;14)(q10;q10) where a monosomic zygote, for instance from a Robertsonian translocation carrier, duplicates the single chromosome. In addition, it could occur through loss of a chromosome in a trisomic zygote, for instance a trisomy produced by a Robertsonian translocation followed by loss of a chromosome (Tomkins *et al.*, 1996). Because enumerator probes cannot differentiate the parental origin of chromosomes, UPD cannot be detected using this approach.

Objectives and outcome of PGD of translocations

Reduction of spontaneous abortions and unbalanced offspring

For most translocation patients, the risk of consecutive pregnancy loss is their major incentive in enrolling in a PGD programme. The unbalanced products of a translocation are usually lethal and therefore the true risk is that of pregnancy loss. We have demonstrated that PGD of translocations substantially increases a couple's chances of sustaining a pregnancy to full term (Munné *et al.*, 1998e, 2000). In the last review of 35 PGD translocation patients, a significant decrease in spontaneous abortions was observed ($P < 0.001$): from 81% of the pregnancies in natural cycles, to 13% in PGD cycles (Munné *et al.*, 2000).

Translocation carriers wish to prevent the disturbing recurrent miscarriages and to sustain pregnancy to full term. We believe that growing embryos to blastocyst stage, as suggested by Menezo *et al.* (1997), cannot select against unbalanced embryos, because many of them implant and are later spontaneously aborted. Similarly, a recent study by Evsikov *et al.* (2000) showed that unbalanced embryos reach blastocyst stage at similar rates as found for chromosomally normal or balanced embryos.

Prevention prognosis depends on several factors

So far, out of 35 pregnancies, six were spontaneously aborted (three balanced and three unknown), one was terminated because undiagnosed embryos were transferred and an unbalanced pregnancy followed, and 38 were delivered. Of these, 37 were normal (71%) or balanced (26%), but one was unbalanced (3%) (data up to September 2001, Munné *et al.*, unpublished).

As explained above, this unbalanced pregnancy was the result of using two telomeric and one centromeric probe. A FISH

error affecting one of the three probes might have resulted in this misdiagnosis, although it might have been the consequence of mosaicism.

Pregnancy prognosis depends on several factors

We found a very good correlation between percentage of chromosomal abnormalities and pregnancy rate. For instance, cases with >50% abnormal eggs or embryos achieve significantly fewer pregnancies per cycle than cases with <50% abnormal eggs or embryos (Munné *et al.*, 2000). This situation arises because most female carriers are fertile and achieve pregnancy easily with normal embryos. In agreement with our observations, other reports on PGD for translocations also showed high rates of abnormal embryos, and none of them resulted in pregnancy in any reported cases (Conn *et al.*, 1998; Van Assche *et al.*, 1999).

Cases involving Robertsonian translocations achieve significantly higher pregnancy rates (50%) than cases involving reciprocal translocations (21%, $P < 0.03$) (Munné *et al.*, 2000b). This is because more abnormal gametes, and therefore abnormal embryos, are produced in reciprocal translocations than in Robertsonian translocations (Munné *et al.*, 2000b). Another series of PGD of translocations also detected more normal embryos in Robertsonian translocation cases than in reciprocal cases, but the series was too small to detect differences in pregnancy rates (Fridstrom *et al.*, 2001).

In patients with reciprocal translocations, the production of unbalanced gametes is likely to occur as a consequence of two mechanisms. One involves meiotic crossing over, and the critical region between the centromere and the breakpoint. The other arises through abnormal meiotic segregation. By contrast, Robertsonian translocation results in unbalanced gametes only as a consequence of abnormal meiotic segregation, since there is no critical region. For instance, analyses of sperm chromosome in patients with Robertsonian translocations have revealed between 0 and 25% abnormal gametes (reviewed by Escudero *et al.*, 2000a), whereas in patients with reciprocal translocations this proportion ranges between 18.4 and 72.1% (reviewed by Estop *et al.*, 1996). Regarding oocytes, significantly more chromosomally abnormal oocytes (71%) arose in reciprocal cases than in Robertsonian translocation cases (42%, $P < 0.001$) (Escudero *et al.*, 2000b; Munné *et al.*, 2000, 2001).

Some reports on translocation carriers indicate a high rate of mosaicism (Conn *et al.*, 1998, Fridstrom *et al.*, 2001). However, Escudero *et al.* (2000b) found similar rates of chromosome abnormalities in both spermatozoa and embryos among Robertsonian carriers.

Sperm analysis as a prognosis tool

Previous studies of segregation modes have been based on post-zygotic material, and have been used to formulate rules to predict unbalanced offspring (Jalbert *et al.*, 1988; Smith and Gaha, 1990). However, the specimens used in these studies came from fetuses and aborted fetuses. These specimens probably showed only the most viable segregation types because selective processes had already occurred. Thus, when

analysing zygotes and pre-implantation embryos, it is not surprising that different translocations involving the same chromosomes show very different meiotic behaviour (Escudero *et al.*, 2000a). Even non-related cases with the same translocation can do the same (Van Assche *et al.*, 1999).

As shown above, pregnancy rates are inversely proportional to the number of abnormal gametes (Munné *et al.*, 2000a). It is highly desirable for prospective patients to know their chances of conception in advance, because the procedures of IVF and PGD are economically daunting and medically complex. One of the principal factors affecting their chances is the percentage of abnormal gametes.

In the study by Escudero *et al.* (2002), an attempt was made to determine the existence of a correlation between chromosome abnormalities in spermatozoa and embryos. If this could be done, it would be useful to determine the level of chromosome abnormalities in spermatozoa that would preclude a chromosomally normal conception. We analysed spermatozoa and all embryos of 11 patients undergoing PGD for reciprocal translocations. A total of 11,184 spermatozoa and 93 embryos were analysed from the 11 patients included in the study. Comparison of FISH sperm analyses using FISH or PGD revealed no statistical difference between segregation types observed in spermatozoa and embryos. The percentages of abnormal gametes and of abnormal embryos were correlated. A predictive equation is proposed for this relationship: $A = -0.55 + (1.9 \times B)$, where A is the fraction of abnormal embryos and B the fraction of abnormal spermatozoa. A total of 16 embryos were replaced in nine of these 11 cases. Four patients became pregnant; three are ongoing and one has delivered a healthy normal baby.

Therefore, Escudero *et al.* (2002) have established that patients with 65% or fewer chromosomally abnormal spermatozoa have a good chance at conceiving. Patients with higher rates will have to produce 10 or more good quality embryos to have reasonable chances of conception.

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