

Article

Preimplantation genetic diagnosis of numerical abnormalities for 13 chromosomes



Dr Santiago Munné

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 the PGD team has shown higher pregnancy rates in women of advanced age undergoing PGD. This team has performed more than 250 PGD cycles for translocations and over 1600 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.

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Abstract

Several types of FISH protocols for PGD have been used to maximize results from a limited number of fluorochromes to study as many chromosomes as possible. The major purpose of the present study was to optimize the use of three sequential hybridizations to analyse up to 15 chromosome types in single cells. A secondary purpose was to study the frequency of aneuploidy of other chromosomes not yet extensively studied in preimplantation embryos. Patients underwent PGD of aneuploidy, and the biopsied cells were analysed with three sequential hybridizations, the first for chromosomes 13, 16, 18, 21 and 22, the second for X, Y, 15 and 17 and the third for 2, 3, 4 and 11. Overall, only 27% of embryos were normal. The chromosomes most involved in aneuploidy were, in order, chromosome 16, 15, 21, 22, 13, 18, 17, 3, 2, 4, 11, and gonosomes. Of the abnormal embryos, only 3% would have been missed without the third set of probes. This protocol allows the simultaneous analysis of up to 15 chromosomes although only 13 were analysed in this study. Results so far show that the chromosomes most involved in abnormalities are those already covered with the two first sets of probes.

Keywords: mosaicism, recurrent miscarriage, trisomy 2, trisomy 3, trisomy 4, trisomy 11

Introduction

Preimplantation genetic diagnosis (PGD) of aneuploidy (Munné *et al.*, 1993; Verlinsky and Kuliev, 1996; Munné, 2002) is a well-established technique used as an alternative to prenatal diagnosis and to improve the success rate of IVF (Gianaroli *et al.*, 1999; Munné *et al.*, 1999). More than 1000 babies have been born so far from this procedure and it is currently applied not only to women of advanced maternal age, but also for patients with recurrent spontaneous abortions (Pellicer *et al.*, 1999), repeated IVF failure (Gianaroli *et al.*, 1999; Kahraman *et al.*, 2000), and non-obstructive azoospermia (Silber *et al.*, 2003).

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.*, 1992; Munné *et al.*, 1995, 1998a). 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. Therefore new colours have been developed, such as Spectrum Gold and Spectrum Blue (Vysis). 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; but still only five chromosomes can be analysed simultaneously.

Nevertheless, once cells are analysed for one set of chromosomes, they can be re-analysed with a different set of probes for a second set of chromosomes (Benadiva *et al.*, 1996; Martini *et al.*, 1997; Bahçet *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 allows the analysis of up to 10 chromosomes simultaneously in a single interphase nucleus in a time frame compatible with regular IVF (Munné *et al.*, 1998b; Gianaroli *et al.*, 1999; 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 in their studies dropped below 80% by the third hybridization, and their protocols were not applied clinically.

Using comparative genome hybridization (CGH), all chromosomes can be analysed in a single cell and this technique has already been used in PGD (Wells *et al.*, 1999; Voullaire *et al.*, 2002). However, the process is extremely time consuming and not suitable for full analysis of a large number of non-replaced embryos in order to obtain meaningful scientific data. In addition, in order to use CGH for PGD purposes, the technique still requires embryo freezing after biopsy, which may jeopardize the survival of the embryo after thawing and preclude the benefits of PGD.

One of the purposes of the present study was to optimize the use of three sequential hybridizations for the analysis of 13–15 chromosome types in a single cell for clinical PGD of numerical chromosome abnormalities.

Many studies have already reported analysis of chromosomes XY, 1, 4, 6, 7, 13, 14, 15, 16, 17, 18, 21 and 22 in considerable numbers of preimplantation embryos and oocytes by fluorescence in-situ hybridization (FISH). Other chromosomes such as 2, 3 and 11 have received scant attention, so another purpose of this study was to determine if they are important in early cleavage stage embryo wastage.

Materials and methods

Patients

Women undergoing PGD of aneuploidy at The Institute for Reproductive Medicine and Science at Saint Barnabas Medical Centre (Livingston, NJ, USA), were 35 years or older, had a history of repeated spontaneous abortion, a previous trisomic conception, or repeated IVF failure. On day 3 of development, each embryo had one cell biopsied (Grifo, 1992), unless that cell did not produce a nucleus, in which case a second one was biopsied. Normally developing embryos classified as chromosomally normal by PGD were transferred to the patient. Some non-transferred embryos were re-analysed with all or most of their cells fixed individually as described previously (Munné *et al.*, 1996), but others with grossly abnormal cells were not re-analysed. Patients undergoing PGD did so in accordance with guidelines approved by the respective internal review boards, including prior written consent from each patient.

Fluorescence in-situ hybridization (FISH) procedure

Fixed cells were analysed by three rounds of FISH. The probes used in each round of hybridization are described in **Table 1**. The first hybridization used the MultiVysion™ PB multicolour

probe panel hybridization mixture commercialized by Vysis (Downers Grove, IL, USA), while the other three panels were prepared in-house as described previously (Munné *et al.*, 1998b; Bahçe *et al.*, 2000). All individual probes as well as the cocktail probe for 13, 16, 18, 21 and 22 were obtained from Vysis.

Of the three multiplex sets described in **Table 1**, the one involving chromosomes 13, 16, 18, 21 and 22 is commercially available (Vysis) and has been properly tested by the provider (Tepperberg *et al.*, 2001). Multiplex probe sets mixed by our laboratory were tested prior to use according to the standards and guidelines for clinical laboratories of the American College of Medical Genetics, with error rate ranges of 1–2% per cocktail on normal cells.

For the first hybridization, 10 µl of the first hybridization solution was applied to the glass slide containing fixed blastomeres and covered with an 18 × 18 mm coverslip. The slide was then placed for 5 min on a slide warmer preheated to 73°C, sealed with rubber cement, and placed in a dark moist chamber at 37°C for 3 h. After hybridization, the slides were washed individually at 71°C in 0.7× concentration standard saline citrate (SSC) for 4 min. The slides were then mounted with 10 µl of antifade solution and observed with a fluorescence microscope (Olympus BX70) equipped with single-band pass filters for each fluorochrome used. Images were composed with the ISIS v3 Metasystem software.

After analysis of the first set of probes, the slides were washed in 0.7 × SSC at room temperature until the coverslips fell off, dipped in distilled water at 71°C for 10 s, and then dehydrated (70, 85, 100% ethanol, 2 min each). A 10 µl aliquot of the second hybridization solution was applied per slide and the slide was processed in the same way as for the first hybridization, but then was washed for 30 s in 0.7× SSC at 71°C. The washed slides were next mounted with 4',6-diamidino-2-phenylindole (DAPI) in antifade (Oncor, FL, USA) and analysed. The third hybridization procedure was identical to the second. **Figure 1** shows two cells analysed following this protocol.

The criteria followed for scoring FISH signals in a single cell and the criteria to assess FISH errors were as previously described (Munné *et al.*, 1998b). Only type I errors were counted, because they were the ones with consequences for the embryo. Previously published criteria to classify chromosome abnormality types by FISH were also followed, either based on a single cell (Silber *et al.*, 2003) or on the whole embryo (Munné *et al.*, 1998a).

Results

Error rate

The error rate was calculated by comparing initial PGD results ($n = 426$) with re-analysis ($n = 200$). Of the 200 re-analysed embryos, 188 were initially classified as abnormal by PGD but after re-analysis, 20 (10.6%) were reclassified as normal. These errors were four complex abnormal, one polyploidy, and 15 aneuploid embryos that after re-analysis were normal. Of the misdiagnosed aneuploidies chromosomes 22 ($n = 5$), 15 ($n = 3$), 21 ($n = 3$), 17 ($n = 2$), 13 ($n = 2$), 16 ($n = 1$), and

Table 1. Probe panels.

	Locus	Label
<i>Panel 1</i>		
Chromosome 13	RB1 locus gene within 13q14	Spectrum Red
Chromosome 16	D16Z3 (satellite II)	Spectrum Aqua
Chromosome 18	D18Z1 (alpha satellite)	Spectrum Blue
Chromosome 21	D21S259, D21S341, D21S342	Spectrum Green
Chromosome 22	BCR gene locus	Spectrum Gold
<i>Panel 2</i>		
Chromosome X	DXZ1 (alpha satellite)	Spectrum Green
Chromosome Y	DYZ3 (alpha satellite)	1:1 Spectrum Aqua: Orange
Chromosome 15	D15Z1 (satellite III)	Spectrum Aqua
Chromosome 17	D17Z1 (alpha satellite)	Spectrum Orange
<i>Panel 3</i>		
Chromosome 2	D2Z1 (alpha satellite)	Spectrum Red
Chromosome 3	D3Z1 (alpha satellite)	Spectrum Orange
Chromosome 4	D4Z1 (alpha satellite)	Spectrum Green
Chromosome 11	D11Z1 (alpha satellite)	Spectrum Aqua

Table 2. Chromosome abnormalities combining PGD and re-analysis results.

	<i>PGD only</i>	<i>PGD confirmed by re-analysis</i>	<i>PGD not confirmed by re-analysis^a</i>	<i>Total</i>
Normal	85	6	8	99
Aneuploid	78	36	0	114
Aneuploid and mosaic	0	64	3	67
Complex abnormal	52	0	0	52
Mosaic $\geq 3/8$ abnormal	0	57	0	57
Mosaic $< 3/8$ abnormal	0	3	12	15
Polyploid	5	7	0	12
Haploid	6	4	0	10
Total	226	177	23	426

^aThe results shown in this column are those for the re-analysis, not for the incorrect PGD analysis.

gonosomes ($n = 1$) were involved in 17 aneuploid events, with two of the 15 embryos being misdiagnosed as double aneuploid. Of the 12 normal embryos that were re-analysed because they had arrested development, three (25%) were in fact aneuploid after re-analysis, one having a monosomy 2 and trisomy 16, another a trisomy 16, and another a monosomy 15 and X.

Thus, per chromosome, and not correcting for false double aneuploidies, the rate was 2.5% ($n = 5$) for 22, 2% ($n = 4$) for 15, 1.5% ($n = 3$) for 16, 1.5% ($n = 3$) for 21, 1.0% ($n = 2$) for 17, 1.0% for 13 ($n = 2$), 0.5% ($n = 1$) for 2 and 0.5% ($n = 1$) for gonosomes. No errors were found for the other chromosomes.

The total error rate was 12% (23/200). Per panel of probes, the first panel produced 13 errors (6.5%), the second seven errors (3.5%), and the third produced one error (0.5%). Two of chromosomes with LSI probes (21, 22), and two of the chromosomes labelled in Aqua (16, 15), had the most errors, explaining why the first panel had the highest error rate.

Chromosome abnormalities

Although only about half of embryos were re-analysed, the information obtained by putting together initial and re-analysis results is indicative of the most common aneuploidies in the cohort of embryos studied.

Of 426 embryos analysed by PGD, 200 were re-analysed and their results, as well as those of the non-re-analysed embryos, are presented in **Table 2**. Overall, only 23% of embryos were normal, 4% had low levels of mosaicism and could be considered mostly normal, and the rest were abnormal. Of the abnormal ones, 27% were aneuploid, 16% were aneuploid and mosaic (total 43% aneuploidy), 13% were mosaics with $> 3/8$ abnormal cells, 12% were complex abnormal by single cell PGD analysis and were most probably chaotic mosaic embryos, and 5% were polyploid or haploid. Thus, the total rate of aneuploidy was 43% and the total rate of mosaicism, if complex abnormalities were considered also mosaics, was 41%.

Of the 181 aneuploid embryos, 66 had double aneuploidy and nine had triple aneuploidy, for 265 aneuploid events. The

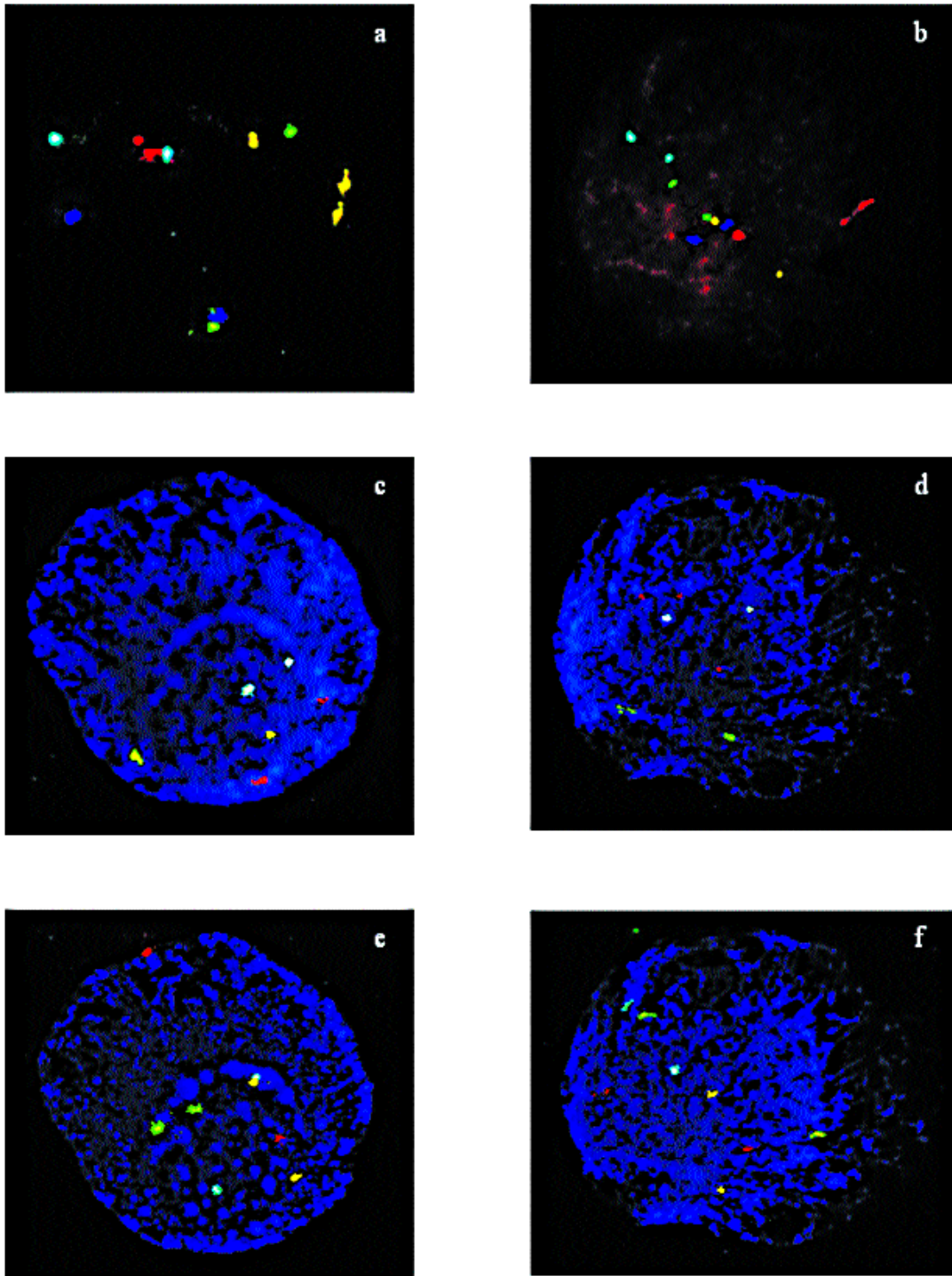


Figure 1. **a, c** and **e** correspond to the first second and third hybridization on the same cell, and **b, d** and **f** to another cell. These two cells were first hybridized with chromosomes 13 (red), 16 (pale blue), 18 (deep blue), 21 (green) and 22 (yellow) as shown in **a** and **b**. **a** depicts a blastomere with trisomy 22, while **b** shows a normal blastomere with a stretched 13 signal. Notice the similar intensity and size of the two chromosomes 22 in close proximity with the other 22 signal (**a**), while the stretched 13 signal in **b** is paler and each portion smaller than the other non-stretched 13 signal. These two cells were subsequently hybridized with probes for chromosomes X (green), Y (pink or aqua: orange mixture), 15 (pale blue), 17 (red) and counterstained with DAPI (**c, d**). Both blastomeres were female but while the one in **c** is normal for these chromosomes, the one in **d** is trisomic for chromosome 17. Finally these two cells were rehybridized with the third set of probes for chromosomes 2 (red), 3 (spectrum orange but here captured as yellow), 4 (green), 11 (pale blue) and counterstained with DAPI (**e, f**). Both blastomeres were normal for these four chromosomes. Overall, the blastomere in **a, c** and **e** had a trisomy 22 and the one in **b, d** and **f** a trisomy 17.

Table 3. Aneuploidy events detected by PGD and/or re-analysis.

Chromosome	PGD	Re-analysis	Total
XY	1	2	3
11	3	6	9
4	4	6	10
2	3	8	11
3	6	5	11
17	9	8	17
18	11	11	22
13	12	11	23
22	15	19	34
21	12	26	38
15	18	23	41
16	20	28	48
Total 1st panel	70	95	165 (62)
Total 2nd panel	28	37	65 (25)
Total 3rd panel	16	25	41 (15)
Total	114	151	265 ^a

^aOf the 181 aneuploid embryos, nine had three aneuploid events after re-analysis, 66 had two events after re-analysis or only PGD (e.g. trisomy 18 and monosomy 21), and the rest had one. Values in parentheses are percentages.

chromosomes most involved in aneuploidy in this study were, in order, chromosomes 16, 15, 21, 22, 13, 18, 17, 3, 2, 4, 11, and gonosomes, as shown in **Table 3**. The error rate of these probes was about 12% (23/(177+23); **Table 2**) and therefore, because only about half the embryos were re-analysed, these frequencies may change slightly the present results.

Third panel chromosomes accounted for 15% of all aneuploidies (**Table 3**). However, because of the high frequency of multiple aneuploidies, the first set of probes correctly identified 82% of all abnormal embryos, including most mosaics, all polyploids and haploids, and first panel aneuploidies. A further 15% were identified by the second probe set for the second chromosome panel; but only 3% required the third probe set to be identified as abnormal.

Discussion

The principle objective of this study was to evaluate the feasibility of using three rounds of hybridization for PGD of aneuploidy in a clinical setting. The two previous studies using three hybridization FISH protocols did not use the protocols clinically (Liu *et al.*, 1998; Vollmer *et al.*, 2000). The 12% type I error rate of the present study is identical to the 12% (15/125) found in a recent study (Magli *et al.*, 2001) using the same first set of probes and a similar second set of probes (with telomeric 21q instead of chromosome 17). The embryos analysed in the present study mostly had morphology and development compatible with replacement and were therefore of generally high quality, which probably minimized errors produced by mosaicism in arrested embryos.

In addition, by analysing first the locus specific probes (13, 21, 22), which are smaller and more prone to misdiagnosis, and satellite DNA probes in the second and third hybridizations, which are larger and more robust, the efficiency of the protocol was probably also increased. By using the five fluorochromes

present in the first panel of probes, it would be possible simultaneously to analyse 15 chromosomes in a single cell using this protocol, in a time compatible with replacement on day 3–5. A full karyotype could be obtained if two cells would be biopsied and each analysed with different probes. However, the biopsy of two cells from an embryo is most probably detrimental for implantation (Tarin *et al.*, 1992). The only study that of PGD results comparing one or two biopsied cells did not compare the same type of embryos (Van de Velde *et al.*, 2000). An alternative to FISH for a whole karyotype is comparative genome hybridization (CGH) (Kallioniemi *et al.*, 1992; Wells *et al.*, 1999), which requires embryo freezing and thawing (Voullaire *et al.*, 2000) to allow enough time for PGD analysis. However, embryo freezing most likely jeopardizes the implantation potential of the biopsied embryos (Magli *et al.*, 1999).

Although in the present study there was only a slight increase in the error rate compared with the previous two-hybridization protocols (usually with about 10% error rate), the extra information obtained by analysing the four chromosomes of the third panel was probably not worth the effort and cost. The rates of aneuploidy for the chromosomes in the third panel accounted only for 15% of all the aneuploidies detected. In addition, these abnormalities accumulated in embryos with double and triple aneuploidies, and so the third hybridization round only found 3% more abnormalities than the first and second panels alone. This may indicate that these chromosomes are only affected by aneuploidy in situations where the oocyte is already seriously compromised. Indeed, oocytes with complex abnormalities are frequently observed by FISH (Verlinsky and Kuliev, 1996; Verlinsky *et al.*, 2001).

That chromosomes 2, 3, 4 and 11 do not contribute much to aneuploidy rates coincides with the data on clinically recognized pregnancies. Major studies have reported that trisomy 16 accounts for 20–35% of all trisomies; acrocentrics and chromosome 2 for 5–10% each; and the rest for less (Hassold *et al.*, 1984; Warburton *et al.*, 1986). Similarly, a previous study with chromosome 4 (Bahçe *et al.*, 1999) also found it rarely involved in aneuploidy, and CGH analysis of embryos derived from repeated IVF failure patients also showed that the probes used in the first two panels would detect 80% of abnormal embryos (Voullaire *et al.*, 2002).

While there is compelling evidence against different survival rates (reviewed by Warburton and Kinney, 1996), others have found that the predominant of aneuploidy types (Schmidt-Sarosi *et al.*, 1998; Bahçe *et al.*, 1999) were different from those found in spontaneous abortions. Until a faster CGH analysis is available that does not require embryo freezing, more studies are needed to ascertain precisely which chromosomes are most frequently involved in embryo wastage. So far, the chromosomes that were used in the first panel and chromosome 15 seem to cause most embryo loss, while the others, when they are involved, usually occur in combination with the most common aneuploidies. It remains to be seen if this is also true for the other chromosomes not analysed in this study, but preliminary data from CGH confirm the present results. A recent study by Voullaire *et al.* (2002) indicates that the 8-chromosome probe FISH test used in PGD (Munné *et al.*, 1998b) can detect about 85% of chromosome abnormalities detected by CGH. This is because these eight

chromosomes are the most involved in aneuploidy, and also because the other chromosome aneuploidies, when they occur, tend to occur simultaneously with aneuploidies involving those eight chromosomes (Voullaire *et al.*, 2002, this study).

In conclusion, single cell biopsy and analysis with the first two sets of probes is currently the most efficient method of performing PGD in a clinical setting, as it provides an acceptably low error rate, is time compatible with regular IVF, and is relatively economical compared with CGH.

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