The analysis of one or two blastomeres for PGD using fluorescence in-situ hybridization

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BACKGROUND: The analysis of one or two blastomeres for PGD using fluorescence in-situ hybridization (FISH) is debated. The proportion of analysable embryos, false negatives, false positives, sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV) and efficiency were evaluated when one or two blastomeres were analysed. METHODS: Embryos of patients having PGD for aneuploidy screening were assigned non-randomly to two groups: group I (n = 413), more slow cleaving embryos with one nucleus for analysis, and group II (n = 1366), regularly cleaving embryos with two nuclei for analysis. A two-round FISH procedure was performed investigating seven chromosomes; 486 embryos were reanalysed. RESULTS: The proportion of analysable embryos was significantly higher in group II (98.2 versus 95.9%) (P = 0.04). Despite the apparently increased false-positive rate (group I: 25.6% and group II: 13.6%) and the decreased PPV (group I: 91.9% and group II: 96.7%), specificity (group I: 74.4% and group II: 86.4%) and efficiency (group I: 93.5% and group II: 97.3%) in group I, no significance was reached (P = 0.11, P = 0.053, P = 0.11 and P = 0.06, respectively). CONCLUSIONS: Although the analysis of one blastomere generates statistically significantly fewer embryos with a diagnosis than does the analysis of two blastomeres, the 2% difference may not be clinically relevant. The diagnostic accuracy is not significantly different between the two groups, hence not favouring the analysis of one or two blastomeres.

Key words: aneuploidy screening/diagnostic accuracy/FISH/one blastomere biopsy/PGD

Introduction
Fluorescence in-situ hybridization (FISH) using multicolour directly labelled probes presents a powerful tool for sexing (Harper et al., 1994; Staessen et al., 1999) and screening numerical (Harper et al., 1995; Munné et al., 1998a) and structural (Munné et al., 2000; Gianaroli et al., 2002) chromosomal abnormalities in embryos to be selected for transfer in assisted reproductive technologies (ART) using PGD.

Because the final goal of PGD is the birth of a healthy child, the diagnostic FISH technique for the enumeration of chromosomes must be efficient, accurate and reliable. High efficiency rates have been reported in the literature after first round of FISH, ranging between 95.2 (Staessen et al., 1999) and 97% (Harper et al., 1994; Munné et al., 1995). An efficiency rate of 95% is reported for single blastomeres when reprobing for a second round (Munné et al., 2003).

However, it is still a matter of discussion whether one or two blastomeres should be analysed for PGD. Analysing two cells per embryo may improve the accuracy but may have a detrimental effect on the developmental and implantation capacity of an embryo (Tarin et al., 1992; Magli et al., 2001). On the contrary, if only a single blastomere is analysed, there is more likelihood that the result may not represent the chromosomal content of the remaining embryo leading to misdiagnosis (Staessen et al., 1999; Ruangvutilert et al., 2000; Van de Velde et al., 2000) and a correct interpretation of the FISH signals on one cell is not always evident. FISH false-positive (embryos diagnosed as abnormal but normal after reanalysis) and false-negative (embryos diagnosed as normal but abnormal after reanalysis) could have clinical relevance either by reducing the number of transferable embryos (false positive) or by transferring aneuploid embryos that are able to implant (false negative) (Gianaroli et al., 1999). Furthermore, scoring errors of FISH signals may arise from loss of nuclear material, overlapping signals, split signals, diffused signals, hybridization failure and probe inefficiency (Ruangvutilert et al., 2000). Besides FISH technique-related errors, chromosomal mosaicism in cleavage-stage embryos could also be responsible for a misdiagnosis. During in vitro preimplantation development, the percentage of chromosomal mosaic embryos increases to almost 100% (ranging from 2 to 90%) at the blastocyst stage (Munné et al., 1994; Magli et al., 2000). A varying incidence
of chromosomal mosaicism in cleavage-stage embryos has been described and depends on maternal age, method of analysis [FISH or comparative genomic hybridization (CGH)], number of chromosomes investigated and definition of mosaicism (Baart et al., 2000; De Vos and Van Steirteghem, 2001; Joris et al., 2003).

So far, one study regarding the accuracy of PGD–FISH has revealed no significant differences between the analyses of one or two blastomeres on a small number of embryos (23 for 1-cell biopsy and 41 for 2-cell biopsy) (Emiliani et al., 2004). The present study was carried out on a much larger number of embryos to report on the proportion of analysable embryos, sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV) and efficiency after a two-round FISH procedure, when one or two blastomeres were analysed, to estimate the impact of the analysis of one blastomere on the accuracy of the genetic test.

Materials and methods

Patients, ovarian stimulation and embryo development

From October 2000 to December 2002, 229 patients underwent PGD for aneuploidy screening, resulting in 313 cycles with 1888 embryos (3717 blastomeres) for biopsy. The number of biopsied embryos per cycle ranged between 1 and 21 embryos. Ovarian stimulation was achieved as previously described (Kolibianakis et al., 2002, 2003). Oocytes were retrieved 36 h after administration of hCG; surrounding cumulus and corona cells were removed, and the nuclear maturation was assessed using an inverted microscope. Only metaphase II (MII) oocytes were injected, and normal fertilization was confirmed by the presence of two distinct pronuclei. Embryonic development was further evaluated on days 2 and 3.

Embryo biopsy

Embryos reaching at least the 5-cell stage on day 3 of development were biopsied as previously described (Van de Velde et al., 2000; De Vos and Van Steirteghem, 2001; Joris et al., 2003).

One blastomere was removed, if the embryo contained five cells. Two blastomeres were biopsied from the 6-cell stage onwards. The definition of a successful biopsy was the removal of a cell without lysis, so that the cell could be used for fixation and analysis (Joris et al., 2003). If none of the blastomeres were successfully biopsied or fixed, a third blastomere was taken, but only if the embryo initially had ≥7 blastomeres (Van de Velde et al., 2000).

Blastomere fixation and FISH procedure

The individual blastomeres of an embryo were fixed on the same slide according to the previously described HCl/Tween 20 method (Coonen et al., 1994; Staessen et al., 1996).

A two-round FISH procedure was performed, which allowed for the detection of chromosomes X, Y, 13, 18 and 21 (Multivision PGT Probe Panel; Vysis, Downers Grove, IL, USA) in the first round and chromosomes 16 and 22 in the second round. The hybridization solution for the second round was prepared by mixing a probe for chromosome 16 (Vysis, Satellite II DNA/D16Z3 probe, Spectrum Orange) and a probe for chromosome 22 (Vysis, LSI 22, 22q11.2, Spectrum Green). The FISH procedure was performed as previously described (Staessen et al., 2003). The results of the first and second round were analysed by two observers, who arrived at consensus on the diagnosis, using a Zeiss Axioskop fluorescence microscope. Images of the blastomeres of the first and second round were captured and compared to ensure that the signals from the first round had not persisted.

FISH scoring criteria

At diagnosis, embryos were considered normal when two gonosomes and two chromosomes 13, 16, 18, 21 and 22 were present; were considered trisomic or monosomic if, respectively, an extra or a missing signal was observed and were considered haploid, triploid or polyplid if, respectively, one, three or more copies of the set of chromosomes were present. An embryo was discordant if one blastomere was defined as normal and the other blastomere as abnormal. Discordant embryos involving a trisomy were always considered abnormal because the disomic cell may be due to a FISH error or loss of nuclear material, giving rise to an abnormal child. Discordant embryos involving a monosomy of chromosomes 13, 18 or 21, which were possibly the result of a FISH error or loss of genetic material, were nevertheless always considered abnormal, because it was considered that, as a result of non-disjunction, the trisomic cell line could also be present. However, discordant embryos including a monosomy 16 or 22 were considered transferable (if no other normal embryos were available and if the patient agreed to have the embryo transferred) because the monosomy could have been due to a FISH error or loss of nuclear material, and if the trisomy cell line is present, the embryo is not viable. Signals were considered as split if the two spots were one, or less than one, domain apart from each other.

Embryos that were abnormal at PGD and after reanalysis are true negatives, and normal embryos at PGD and after reanalysis are true positives. If an embryo was abnormal at PGD and normal after reanalysis, it was considered transferable (if no other normal embryos were available and if the patient agreed to have the embryo transferred) because the monosomy could have been due to a FISH error or loss of nuclear material, giving rise to an abnormal child. Discordant embryos involving a trisomy were always considered abnormal.

Table I. Comparison of the results of a diagnostic test (PGD) with the “true” status after reanalysis (“gold standard”)

<table>
<thead>
<tr>
<th>Test result PGD</th>
<th>Reanalysis = truth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal</td>
<td>True positive (A)</td>
</tr>
<tr>
<td>Normal</td>
<td>False negative (C)</td>
</tr>
<tr>
<td>Abnormal</td>
<td>False positive (B)</td>
</tr>
<tr>
<td>Normal</td>
<td>True negative (D)</td>
</tr>
</tbody>
</table>

Reanalysis

All the patients gave written consent for research on their embryos not suitable for transfer or cryopreservation. Genetically abnormal embryos or genetically normal embryos, which were not transferred or frozen, were reanalysed to confirm the initial diagnosis. The embryos were fixed on day 5 or 6, and FISH was performed using the same probe set as at initial diagnosis. The diagnosis was uniformly confirmed if the abnormality or normality detected at diagnosis was confirmed in at least 80% of the nuclei after reanalysis. If the abnormality or normality detected at diagnosis was present in at least 10% of the nuclei after reanalysis, the cell line was considered as present in the embryo after reanalysis, and the diagnosis was also considered confirmed (Sandalinas et al., 2001; Baart et al., 2004).

Statistical analysis

In this study, the outcome variables were the proportion of analysable embryos (with result/without result), the diagnosis of the embryo at PGD (normal/abnormal) and the diagnosis after reanalysis of the embryos (normal/abnormal). Table I was constructed after reanalysis of the embryos.
reanalysis, this is a false positive. If the diagnosis was normal at PGD and abnormal after reanalysis, this is a false negative. Based on this table, the following parameters of a test can be calculated: the sensitivity \([A/(A + C)] \times 100\), the specificity \([D/(B + D)] \times 100\), the false-negative rate \([C/(A + C)] \times 100\), the false-positive rate \([B/(B + D)] \times 100\), the NPV \([D/(C + D)] \times 100\) and the PPV \([A/(A + B)] \times 100\). In addition, the table also enables us to calculate the efficiency of the technique \([\{(A + D)/(A + B + C + D)\} \times 100\].

The results of the proportion of analysable embryos, falsely normal and falsely abnormal results and the statistical parameters such as sensitivity, specificity, false-negative rate and false-positive rate were evaluated in both group I (one nucleus for analysis) and group II (two nuclei for analysis). However, these results should not be determined from the data pooled over all embryos because the data concern several embryos from the same patient during the same cycle. Therefore, a statistical technique is to be used which takes the clustered nature of the data into account. Generalized estimating equation (GEE) modeling, allowing logistic regression with repeated measures, is the appropriate technique (Liang and Zeger, 1986). GEE is a method that models binary and count data, taking into account the correlation between data arising from repeated measurements. The method fits a generalized linear model to the data by maximizing the likelihood estimation of the vector of dependent variables. The fitting is performed through an iterative fitting process. This statistical test is two tailed and applied to all the results presented in this article. The results were considered significant if \(P < 0.05\).

Results

Proportion analysable embryos

A total of 1888 embryos (3717 blastomeres) were available for biopsy (Figure 1). Two blastomeres were biopsied in 1711 embryos (90.6%); one cell was biopsied in 118 embryos (6.3%), and a third cell was biopsied in 59 embryos (3.1%). This resulted in 1779 (94.2%) successfully biopsied embryos or 3687 (99.2%) successfully biopsied blastomeres. Of the 1779 successfully biopsied embryos, 102 embryos (5.7%) were at the 5-cell stage on day 3, 280 embryos (15.7%) were at the 6-cell stage, 338 embryos (19.0%) were at the 7-cell stage, 727 embryos (40.9%) were at the 8-cell stage, 220 embryos (12.4%) had more than eight blastomeres and 112 embryos (6.3%) were compacted on day 3. A total of 20 blastomeres (0.5%) were lost during fixation; 107 (2.9%) were anucleated; 56 (1.5%) contained a metaphase; 319 (8.6%) had multiple or fragmented nuclei and 53 (1.4%) had a nucleus from a lysed blastomere. One distinct nucleus was observed in 3132 (84.9%) of the blastomeres.

The proportion of analysable embryos after the first and second round is summarized in Table II. Two categories were identified non-randomly: group I, embryos with one nucleus for analysis (embryos with one blastomere successfully biopsied and fixed or two or three blastomeres biopsied but only one blastomere with a single spread nucleus available), and group II, embryos with two nuclei available for analysis (embryos with two or three blastomeres biopsied and two single spread nuclei available). Group I consisted of more slow cleaving 5- or 6-cell embryos than group II (37 and 17% for groups I and II, respectively). From group I \((n = 413)\), 400 embryos (96.9%) was analysable after the first round. No or no reliable result was available in 13 blastomeres because of procedure-related factors: (i) a nucleus lost during the procedure in two cases (0.5%), (ii) a damaged or an incomplete nucleus in three cases (0.7%) and (iii) debris on the nucleus in eight cases (1.9%). After the second round, 396 embryos (95.9%) had an analysable result with a result for at least one chromosome, that is, either chromosome 16 or chromosome 22. For four embryos, no or no reliable result was obtained due to the following factors: (i) a nucleus lost during procedure in one case (0.5%), (ii) debris on the nucleus in two cases (0.5%) and (iii) no signals in one case (0.2%). Group II contained 1366 embryos, of which 1356 (99.3%) produced an analysable result after the first round. This is significantly higher than in group I \((P = 0.02)\). In 8.7% of the embryos, the diagnosis of the first round was based on one nucleus, although two blastomeres were available for analysis. In 10 cases, no or no reliable result was available due to the following factors: (i) both nuclei lost during procedure in one case (0.1%), (ii) both nuclei damaged in one case (0.1%), (iii) both nuclei with debris in six cases (0.4%), (iv) both nuclei without signals in one case (0.1%) and (v) a combination of the previous categories in one case (0.1%). A diagnosis of the second round was obtained in 1341 embryos (98.2%), which is also significantly higher than in group I \((P = 0.04)\). In 11.7% of the embryos, the result was based on one nucleus. No or no reliable result was obtained in 15 cases due to the following factors: (i) both nuclei lost during procedure in one case (0.1%), (ii) both nuclei damaged in one case (0.1%), (iii) both nuclei with debris in three cases (0.2%) and (iv) the absence of signals in 10 cases (0.7%).

False-normal and false-abnormal results after reanalysis

Table III summarizes the data after reanalysis of 486 embryos, which were abnormal or normal and not transferable or frozen. Embryos were reanalysed on day 5 or 6, but most of them were reanalysed on day 6 (group I: 89.5% and group II: 91.3%).

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**Figure 1.** Flow chart of embryos/blastomeres from biopsy until blastomere fixation.
FISH analysis of one or two blastomeres for PGD

reanalysis of 157 embryos from group II, of which the diagnosis was based on one blastomere after two rounds although two blastomeres were available for analysis (see table II), was not included in the data. From the 86 reanalysed embryos (29 of group I and 57 of group II), which were normal at PGD, all 86 were confirmed, not revealing any embryos falsely diagnosed as normal. The remaining 400 reanalysed embryos (124 in group I and 276 in group II) were diagnosed as abnormal at PGD. A total of 8.1% (10/124) of the embryos was falsely diagnosed as abnormal in group I. Of those 10 cases, falsely diagnosed as abnormal, one embryo was diagnosed as trisomy 18, two embryos were monosomic (monosomy 22 and monosomy 13), one was combined abnormal embryo and five were discordant embryos (two monosomy 16/disomy 16 embryos, one monosomy 21/disomy 21 embryo, one monosomy X/disomy X embryo and one normal/combined abnormal embryo). Monosomies were involved in four of five not confirmed discordant embryos. The proportion of embryos that were falsely diagnosed as abnormal (8.1% for group I and 3.3% for group II) is higher in the 1-cell group but not significantly different (*P = 0.053).

Table II. Proportion of analysable embryos after the first and second round: the analysis of one (group I) versus two blastomeres (group II)

<table>
<thead>
<tr>
<th>Number of blastomeres for analysis</th>
<th>Group I (one blastomere)</th>
<th>Group II (two blastomeres)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of embryos</td>
<td>413</td>
<td>1366</td>
<td>1779</td>
</tr>
<tr>
<td>Number of analysable embryos after first round (%)</td>
<td>400 (96.9)*</td>
<td>1356 (99.3)*</td>
<td>1765 (98.7)</td>
</tr>
<tr>
<td>Based on one blastomere (%)</td>
<td>400</td>
<td>118 (8.7)</td>
<td>518 (29.5)</td>
</tr>
<tr>
<td>Based on two blastomeres (%)</td>
<td></td>
<td>1238 (91.3)</td>
<td>1238 (70.5)</td>
</tr>
<tr>
<td>Number of analysable embryos after second round (%)</td>
<td>396 (95.9)*</td>
<td>1341 (98.2)*</td>
<td>1757 (97.6)</td>
</tr>
<tr>
<td>Based on one blastomere (%)</td>
<td>396</td>
<td>157 (11.7)</td>
<td>513 (31.8)</td>
</tr>
<tr>
<td>Based on two blastomeres (%)</td>
<td></td>
<td>1184 (88.3)</td>
<td>1184 (68.2)</td>
</tr>
</tbody>
</table>

Generalized estimating equation (GEE) modelling: significant difference (*P = 0.02, *P = 0.04).

Table III. Results after reanalysis evaluating the false-normal and false-abnormal results between a diagnosis based on one and on two blastomeres

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Group I (one blastomere)</th>
<th>Group II (two blastomeres)</th>
<th>Number of reanalysed</th>
<th>Number of confirmed</th>
<th>Number of not confirmed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>29</td>
<td>29</td>
<td>0</td>
<td>57</td>
<td>57</td>
</tr>
<tr>
<td>Falsely normal: 0% (0/29)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abnormal</td>
<td>124</td>
<td>114</td>
<td>10</td>
<td>267</td>
<td>267</td>
</tr>
<tr>
<td>Falsely abnormal: 8.1% (10/124)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trisomy</td>
<td>29</td>
<td>25</td>
<td>4 (13.8%)</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>Monosomy</td>
<td>39</td>
<td>35</td>
<td>4 (10.2%)</td>
<td>61</td>
<td>59</td>
</tr>
<tr>
<td>Haploid/triploid/polyploid</td>
<td>7</td>
<td>6</td>
<td>1 (14.3%)</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Combined abnormal</td>
<td>49</td>
<td>48</td>
<td>1 (2.0%)</td>
<td>111</td>
<td>110</td>
</tr>
<tr>
<td>Discordant</td>
<td></td>
<td></td>
<td></td>
<td>58</td>
<td>53</td>
</tr>
<tr>
<td>Falsely abnormal: 3.3% (9/276)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Generalized estimating equation (GEE) modelling: not significant (*P = 0.053).

Table IV. Diagnostic accuracy after reanalysis, evaluating a diagnosis based on one and two blastomeres

<table>
<thead>
<tr>
<th>PGD result</th>
<th>Group I (one blastomere)</th>
<th>Group II (two blastomeres)</th>
<th>Abnormal</th>
<th>Normal</th>
<th>Abnormal</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>114</td>
<td>0</td>
<td>267</td>
<td>9</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100% (114/114)</td>
<td>100% (267/267)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>74.4% (29/39)*</td>
<td>86.4% (57/66)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>False-negative rate</td>
<td>0% (0/114)</td>
<td>0% (0/267)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>False-positive rate</td>
<td>25.6% (10/39)*</td>
<td>13.6% (9/66)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>100% (29/29)</td>
<td>100% (57/57)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>91.9% (114/124)*</td>
<td>96.7% (267/276)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Efficiency</td>
<td>93.5% (143/153)*</td>
<td>97.3% (324/333)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Generalized estimating equation (GEE) modelling: no significant differences (*P = 0.11, *P = 0.11, *P = 0.053 and *P = 0.06).
in both groups), the false-negative rate (0% in both groups) and NPV (100% in both groups). The specificity (group I: 74.4% and group II: 86.4%), the false-positive rate (group I: 25.6% and group II: 13.6%), the PPV (group I: 91.9% and group II: 96.7%) and efficiency (group I: 93.5% and group II: 97.3%) were not significantly different for the two groups (P = 0.11, P = 0.11, P = 0.053 and P = 0.06, respectively). None of the parameters associated with the diagnostic accuracy were significantly different, hence not favouring the analysis of one or two blastomeres.

Discussion
It is still a matter of debate whether to analyse one or two blastomeres for PGD–FISH. Three aspects of the current study will be discussed in more detail: the proportion of analysable embryos, false-normal and false-abnormal results after reanalysis and the diagnostic accuracy.

The difference in the proportion of analysable embryos between the analyses of one or two blastomeres is significantly higher (group I: 95.9% and group II: 98.2%) if two blastomeres are analysed (P = 0.04). This rate is similar to the 95% efficiency rate reported by Munné after second round FISH (Munné et al., 2003). This indicates an advantage when two blastomeres are available for analysis; there is a greater chance of achieving a result based on one nucleus when the other blastomere is not interpretable (mainly because damaged or debris). Another advantage is that when the result is doubtful in one nucleus (split spot), the result of the second cell might be convincing. Although this difference is significant, the proportion of 95.9% of embryos with result when one blastomere is analysed is acceptable, and the 2% difference may not be clinically important.

Reanalysis of non-transferred embryos at day 5 or 6 is a useful tool for investigating whether the initial result, obtained at day 3, is confirmed and is representative for the whole embryo. In this study, no embryos were falsely diagnosed as normal in either of the two groups. However, several false-normal results after reanalysis with 1-cell analysis [Sandalinas et al., 2001 (1.5%) and Munné et al., 1999b (9.0%)] and 2-cell analysis [Baart et al., 2004 (5.9%)] have been reported. In our study, the proportion of embryos falsely diagnosed as abnormal after reanalysis was higher if one nucleus was analysed (8.1 versus 3.3%), though not significantly (P = 0.053). The result of 8.1% of embryos, falsely diagnosed as abnormal embryos after 1-cell analysis, is similar to the results in the literature [Sandalinas et al., 2001 (7.0%) and Munné and Weier, 1996 (14%)]. Considering the embryos that were falsely diagnosed as abnormal if two blastomeres were analysed (3.3%), an interesting observation is that five of nine embryos involved discordant results at diagnosis. One explanation may be that nuclear material was lost in one blastomere. Another possibility is a FISH scoring error such as, an overlap of two homologue chromosomes or a misinterpretation of split spots. Another explanation may be related to mosaicism in an embryo. Abnormal cells from a mosaic embryo can be eliminated by apoptosis (Hardy, 1997; Bielanska et al., 2002; Coonen et al., 2004) and could result in a normal embryo after reanalysis on day 5 or 6. In this study, one discordant embryo monosomy 16/disomy 16 was transferred (patient agreed and there were no other normal embryos available), but no pregnancy was obtained. From the embryos available for reanalysis, this study revealed a higher proportion of embryos falsely diagnosed as abnormal when one nucleus was analysed, but no significance was reached (P = 0.053). No embryos were falsely diagnosed as normal.

Embryo reanalysis also enables several parameters to be determined (sensitivity, specificity, false-negative rate, false-positive rate, NPV, PPV and the efficiency) to evaluate the diagnostic accuracy between the analyses of one or two blastomeres. No differences were observed between the two groups regarding the sensitivity (probability that an embryo is abnormal, given an abnormal result after reanalysis) and NPV (probability that an embryo is normal, given a normal result at PGD). The false-negative rate (proportion of normal embryos at PGD among abnormal embryos after reanalysis) is 0% in both groups [with a 95% confidence interval (CI) (0–2.6%) for 1-cell analysis and 95% CI (0–1.1%) for 2-cell analysis]. None of the other parameters, that is, PPV (probability that an embryo is abnormal, given an abnormal result at PGD), specificity (probability that an embryo is normal, given a normal result after reanalysis) and efficiency, were significantly different between the analyses of one or two blastomeres (P = 0.053, P = 0.11 and P = 0.06, respectively). No significant difference regarding the false-positive rate (proportion of abnormal embryos at PGD among normal embryos after reanalysis) was observed between the two groups (P = 0.11). It should also be noted that false-positive rates of 25.6% (10/39) for group I and 13.6% (9/66) for group II may seem high, but this can be explained because only embryos that were not transferred or frozen were available for reanalysis, which causes an overestimation of the false-positive rates thus calculated. The number of cycles on which the false-positive rate is calculated (39 cycles for group I and 66 cycles for group II) is low, so the chance of demonstrating a significant difference between the two groups is also low (power calculation 11%). More data should be available to provide more valuable information regarding the false-negative and false-positive rates. Most articles have not calculated false-positive and false-negative rates but reported on the proportion of embryos falsely diagnosed as abnormal or normal after reanalysis (Munné and Weier, 1996; Sandalinas et al., 2001). Only one study by Emiliani et al. (2004) similarly reported the results and revealed no significant differences between the analyses of one or two nuclei regarding the accuracy of the FISH technique, not favouring the analysis of one or two nuclei. The FISH efficiency rates of Emiliani et al. (2004) were lower than those in this study (70 and 78% respectively for 1- and 2-cell biopsy), and a false-negative rate of 8% was observed in the 1-cell group, but this may be because of the small number of embryos analysed (23 for 1-cell biopsy and 41 for 2-cell biopsy).

Another approach to evaluate a diagnostic test is to analyse the likelihood ratios (Greenberg et al., 2001). The negative likelihood ratio [i.e. (1 – sensitivity)/specificity] is also 0 in both groups because the first probability was 0% in both groups. The positive likelihood ratio [i.e. sensitivity/(1 – specificity)] is higher in group II (7.35 in group II versus 3.91...
in group I), which indicates that a diagnosis based on two blastomeres produces a higher diagnostic value of the PGD–FISH test than a diagnosis based on one blastomere. With one blastomere evaluated, there were approximately four true positives for every false positive, and, with two blastomeres evaluated, there were approximately seven true positives for every false positive. In addition, likelihood ratios are useful because they are an indication of the degree to which a PGD test result will change the pretest probability of an abnormal embryo. The pretest probability (0.74 for group I and 0.80 for group II) and the post-test probability (0.92 for group I and 0.97 for group II) indicate that a positive test result (i.e. abnormal embryo) of the PGD–FISH procedure moderately increases the probability of an abnormal embryo in both groups.

Regarding the validity of the results, the first important point that needs to be made is that only abnormal or not transferred embryos are available for reanalysis and provide information whether an embryo is correctly rejected for transfer or not. The question is whether these calculated rates are representative for the embryos that have been transferred. So far, no children with obvious numerical chromosomal abnormalities involving one of the tested chromosomes have been observed in our centre after PGD for aneuploidy screening. However, some misdiagnoses involving a trisomy 21 after 1-cell analysis have been reported in the literature (one by Munné et al., 1998a and two by Gianaroli et al., 2001). The European Society of Human Reproduction and Embryology (ESHRE) Consortium also reported three misdiagnoses after PGD–FISH, but no information was provided whether this was after 1- or 2-cell analysis (Sermon et al., 2005; Harper et al., 2006). Another issue that should be mentioned is that group I (the analysis of one blastomere) includes the biopsied 5-cell stage slowly cleaving embryos, whereas the 2-cell group includes more regularly cleaving embryos. Varying rates of genetic abnormalities may be observed between the two possibly distinct populations—i.e. regularly cleaving embryos will have a lower probability of abnormalities—which might result in a potential bias. This study aims at describing the accuracy and predictive value of the FISH technique when one and two blastomeres are analysed and provides no answer whether to biopsy one or two blastomeres. This important question can only be addressed through a randomized controlled study comparing clinical outcome after biopsy of one or two blastomeres.

To conclude, these data provide evidence that the analysis of two blastomeres generates statistically significantly more embryos with a diagnosis after a two-round FISH procedure, but the 95.9% detection rate of embryos with a result after one blastomere analysis is acceptable and the 2% difference in the proportion of analysable embryos may not be clinically important. No significant differences were observed for embryos that were falsely diagnosed as normal or abnormal after reanalysis, and the analysis of one blastomere seems justified in terms of accuracy of the FISH technique. This does not necessarily hold true for other PGD applications by FISH, such as translocations. In general, it should be emphasized that PGD–FISH on one nucleus or two nuclei does not make prenatal diagnosis obsolete.

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