The combination of polar body and embryo biopsy does not affect embryo viability

M. Cristina Magli, Luca Gianaroli, Anna P. Ferraretti, Marco Toschi, Francesca Esposito and M. Carmen Fasolino

S.I.S.Me.R., Reproductive Medicine Unit, Via Mazzini 12, 40138 Bologna, Italy

BACKGROUND: The biopsy of both polar bodies and a blastomere from the same embryo was investigated as an approach aimed at increasing the quantity of DNA available for genetic analysis in preimplantation embryos.

METHODS: In 113 cycles, preimplantation genetic diagnosis (PGD) was performed for aneuploidy: 19 cycles underwent polar body biopsy, 32 cycles had both polar body and blastomere biopsy done, and the remaining 62 cycles underwent blastomere biopsy. The chromosomal analysis was performed in a two-round fluorescence in situ hybridization (FISH) protocol with probes specific for the chromosomes X, Y, 13, 15, 16, 18, 21 and 22. RESULTS: The morphological evaluation of the analysed embryos demonstrated similar rates of development irrespective of the biopsy procedure. Accordingly, the implantation rate did not differ significantly in the three biopsy groups and was 15% after polar body biopsy, 26% after the combined biopsy procedures of polar bodies and blastomeres, and 25% after blastomere biopsy. CONCLUSIONS: The removal of one blastomere subsequent to polar body biopsy does not seem to have negative effects on embryo viability. This approach could be especially valuable for a combined diagnosis of aneuploidy and single-gene disorders in preimplantation embryos generated by couples at high reproductive risk.

Key words: aneuploidy/blastomere biopsy/embryo viability/polar body biopsy/preimplantation genetic diagnosis

Introduction

Preimplantation genetic diagnosis (PGD) for aneuploidy and single-gene disorders on in vitro generated embryos is a recent achievement in reproductive medicine aimed at giving couples with high reproductive risk a better chance of delivering a healthy infant. Following experiments with animal embryos at different stages of development (Gardner and Edwards, 1968; Wilton and Trounson, 1987; Takeuchi et al., 1992), the biopsy of one blastomere from day 3 embryos was proposed in human IVF (Handyside et al., 1990). The procedure entails the breaching of the zona pellucida and the removal of one blastomere from morphologically normal embryos at 62–64 h post-insemination. At this time, intercellular junctions begin to form among blastomeres which leads to compaction. The biopsy is generally performed shortly before this process initiates in order to avoid cellular damage. Although highly invasive, no detrimental effect on further growth has been demonstrated (Hardy et al., 1990). More importantly, data from the clinical outcome of biopsied embryos have shown that approximately a quarter of them are capable of implantation (Gianaroli et al., 1999a; Munné et al., 2003).

An alternative approach is represented by performing PGD on polar bodies; in this way, the integrity of the embryo is preserved as the by-products of the meiotic division are used for genetic analysis (Verlinsky et al., 1990). Unfortunately, neither paternally derived defects nor aneuploidy events generated at fertilization or after the first cleavage divisions can be diagnosed (Kuliev et al., 2003).

In view of these considerations, blastomere biopsy permits a more complete genetic diagnosis of the embryo even though, in the case of aneuploidy, mosaicism could cause misdiagnosis at a rate of ~5% (Gianaroli et al., 2001; Munné et al., 2002).

In order to reduce the error rate associated with 1-cell diagnosis, some authors have proposed to biopsy two cells from each embryo and to base the genetic diagnosis on a 2-cell result (Sermon and Libaers, 1999). However, no comparative studies have been done on the implantation rate of embryos after 1- versus 2-cell biopsy, and the maintenance of viability in relation to the reduction of embryonic mass remains a concern. According to our policy, PGD for aneuploidy is routinely performed on one blastomere, as the current error rate is considered to be acceptable with the present pregnancy rate (Gianaroli et al., 1999a, 2001). In addition, a retrospective analysis of the clinical outcome after 1-cell biopsy indicates that 29% of the embryos transferred are able to implant, whereas after 2-cell biopsy the implantation rate drops to 16%. This calculation was done in the case of embryos with at least...
six cells at the time of biopsy. The results obtained clearly indicate that the success of a PGD cycle is a subtle balance between exactness of the diagnosis and preservation of embryo viability. Nevertheless, in cases of extremely high reproductive risk such as for carriers of translocations or single-gene disorders, the decision to remove two cells from each embryo in order to decrease the risk of misdiagnosis is quite acceptable.

The purpose of this study was to evaluate the possibility of an alternative approach aimed at increasing the quantity of DNA available for genetic analysis that can be obtained from a preimplantation embryo. For this reason, both polar bodies and a blastomere were biopsied from the same embryo in patients with a poor prognosis for pregnancy. Embryo morphology, cleavage and implantation rates were monitored in order to establish whether the two combined biopsy procedures could have an effect on embryo viability. The results obtained were compared with those derived from cycles with the same characteristics having either polar body or blastomere biopsy done for aneuploidy screening.

**Materials and methods**

**Patients**

This study included a total of 110 patients with a poor prognosis of pregnancy who underwent 113 cycles of PGD for aneuploidy between June 1999 and July 2003. Chromosomal analysis on the polar body was proposed to 48 of these patients (51 cycles), whereas the remaining 62 underwent PGD for aneuploidy on blastomeres.

The 48 patients programmed for polar body biopsy had a mean maternal age of $38.4 \pm 3.7$ years; the indication for PGD was a poor prognosis due to a maternal age $\geq 38$ years (29 cycles, mean maternal age $41.1 \pm 1.8$ years) or repeated IVF failures (22 cycles, mean maternal age $34.9 \pm 2.4$ years and at least three previous IVF failures).

In the group of advanced maternal age, 27 cycles had already experienced 96 previous IVF failures including 23 unsuccessful attempts of PGD for aneuploidy on preimplantation embryos. The 22 cycles included in the group of repeated IVF failures had already undergone 81 previous IVF cycles, of which 15 were in combination with PGD for aneuploidy on blastomeres.

Sixty-two patients (62 cycles) with the same poor prognosis indications had oocyte retrievals performed on the same day as the 51 cycles programmed for polar body biopsy: 35 patients had a maternal

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**Figure 1.** FISH analysis was performed on polar bodies with the probes specific for chromosomes 13 (red), 16 (aqua), 18 (pink), 21 (green) and 22 (yellow). The first polar body was clearly euploid (A), whereas a diagnosis could not be completed for the second polar body due to pulverization of the signals (B). The same probes were tested on a blastomere biopsied from the corresponding day 3 embryo that was classified as chromosomally normal (C).
age of ≥38 years (mean age 40.5 ± 2.1 years) with a total of 102 previous failed cycles experienced by 34 of them; the remaining 27 patients were <38 years old (mean maternal age 35.0 ± 2.0 years) and had already undergone 88 unsuccessful cycles.

Ovulation induction was performed using a long-term LH-releasing hormone analogue and exogenous gonadotrophins whose dosage was dependent on the ovarian follicular response of the patient (Ferraretti et al., 1996). A single dose of HCG was given to trigger ovulation; 34 h later, transvaginal ultrasound-guided oocyte retrieval was performed and the collected oocytes were incubated in a 5.3% CO₂ humidified gas atmosphere at 37.3°C. Oocyte insemination was performed with ICSI or conventional IVF depending on semen sample indices.

### Polar body and embryo biopsy

At ~16 h after insemination, oocytes were scored for the presence of pronuclei and polar bodies (Gianaroli et al., 2003a). Regularly fertilized oocytes were selected to undergo genetic analysis and the two polar bodies were removed simultaneously. Pronuclear zygotes were biopsied individually in HEPES-buffered medium overlaid with overnight pre-equilibrated mineral oil. Both polar bodies were removed by using a polished glass needle, which was introduced into the perivitelline space after opening a breach of ~20 μm in the zona pellucida with acidic Tyrode’s solution (pH 2.35). After biopsy, pronuclear zygotes were cultured individually and observed at 40, 62 and 88 h post-insemination for embryo development. The number and morphology of nuclei and blastomeres, and the percentage of fragmentation were documented.

Blastomere biopsy was performed on day 3 embryos with regular morphology and development at 62–64 h after insemination. Embryos were biopsied individually in HEPES-buffered medium overlaid with overnight pre-equilibrated mineral oil. After loading acidic Tyrode’s solution in a 12 μm diameter pipette, an opening was made in the zona pellucida of ~20 μm diameter. One nucleated blastomere was aspirated into a 30–40 μm diameter glass pipette with extreme care to avoid damage to either the biopsied cell or the surrounding blastomeres. After biopsy, the embryos were washed thoroughly, put in fresh medium and incubated until the time of transfer.

In the case of incomplete diagnosis after chromosomal analysis on polar bodies, regularly developing day 3 embryos were selected for blastomere biopsy (Figure 1). The same procedure was followed with the exception that the blastomere was removed through the same opening made with acidic Tyrode’s solution at the time of polar body biopsy.

### Fluorescence in situ hybridization (FISH)

The biopsied polar bodies were transferred to water, fixed in methanol:acetic acid on a glass slide, and dehydrated in methanol. The biopsied blastomeres were transferred to hypotonic solution, fixed in methanol:acetic acid on a glass slide, and dehydrated in an ethanol series (70, 85 and 100%).

In both cases, probes for the chromosomes X, Y, 13, 15, 16, 18, 21 and 22 were used in a two-round protocol for the simultaneous detection of the chromosomes most frequently involved in spontaneous abortions and trisomic pregnancies (Munné et al., 1998a). Chromosomes 13, 16, 18, 21 and 22 were screened in the first round of hybridization, (Multivision PB panel from Vysis; Vysis Inc., Downers Grove, IL) followed by a second round with probes specific for chromosome X (Vysis, CEP X alpha satellite, Xp11.1-q11.1), Y (Vysis, CEP Y alpha satellite, Yp11.1-q11.1), 15 (Vysis, CEP 15 satellite III, 15q11.2) and 21 (Vysis, Tel 21). The scoring criteria used for the interpretation of FISH signals have been described previously (Munné et al., 1998b; Magli et al., 2001a).

### Embryo transfer and pregnancy outcome

In order to complete the genetic analysis, embryo transfer was scheduled on day 4 (Gianaroli et al., 1999b). Only embryos diagnosed as chromosomally normal were selected for transfer and placed back

### Table I. Overall results in patients with indications for PGD for aneuploidy on the polar body

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>48</td>
</tr>
<tr>
<td>No. of cycles</td>
<td>51</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>38.4 ± 3.7</td>
</tr>
<tr>
<td>Previous IVF cycles</td>
<td>2.0 ± 2.4</td>
</tr>
<tr>
<td>No. of oocytes</td>
<td>526</td>
</tr>
<tr>
<td>No. of 2PN biopsied</td>
<td>346</td>
</tr>
<tr>
<td>FISH normal (%)</td>
<td>77 (22)</td>
</tr>
<tr>
<td>FISH abnormal (%)</td>
<td>183 (53)</td>
</tr>
<tr>
<td>No result (%)</td>
<td>86 (25)</td>
</tr>
<tr>
<td>No. of biopsied embryos</td>
<td>67</td>
</tr>
<tr>
<td>FISH normal (%)</td>
<td>31 (46)</td>
</tr>
<tr>
<td>FISH abnormal (%)</td>
<td>36 (54)</td>
</tr>
</tbody>
</table>

### Table II. FISH and clinical results analysed in relation to the biopsy procedure

<table>
<thead>
<tr>
<th></th>
<th>Polar body</th>
<th>Polar body and blastomere</th>
<th>Blastomere</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cycles</td>
<td>19</td>
<td>32</td>
<td>62</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>38.4 ± 4.0</td>
<td>38.4 ± 3.6</td>
<td>38.1 ± 3.4</td>
</tr>
<tr>
<td>Previous IVF cycles</td>
<td>2.7 ± 2.7</td>
<td>3.9 ± 2.0</td>
<td>3.1 ± 2.3</td>
</tr>
<tr>
<td>No. of oocytes</td>
<td>199</td>
<td>327</td>
<td>634</td>
</tr>
<tr>
<td>No. of 2PN biopsied</td>
<td>108</td>
<td>238</td>
<td>–</td>
</tr>
<tr>
<td>FISH normal (%)</td>
<td>29 (27)</td>
<td>48 (20)</td>
<td>–</td>
</tr>
<tr>
<td>FISH abnormal (%)</td>
<td>69 (64)</td>
<td>114 (48)</td>
<td>–</td>
</tr>
<tr>
<td>No result (%)</td>
<td>10 (9)</td>
<td>76 (32)</td>
<td>–</td>
</tr>
<tr>
<td>No. of biopsied embryos</td>
<td>–</td>
<td>67</td>
<td>376</td>
</tr>
<tr>
<td>FISH normal (%)</td>
<td>–</td>
<td>31 (46)</td>
<td>117 (31)</td>
</tr>
<tr>
<td>FISH abnormal (%)</td>
<td>–</td>
<td>~36 (54)</td>
<td>258 (69)</td>
</tr>
<tr>
<td>No result (%)</td>
<td>–</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>No. of embryos transferred</td>
<td>20</td>
<td>50</td>
<td>76</td>
</tr>
<tr>
<td>No. of cycles transferred (%)</td>
<td>13 (68)</td>
<td>29 (91)</td>
<td>47 (76)</td>
</tr>
<tr>
<td>No. of clinical pregnancies (%)</td>
<td>3 (23)</td>
<td>10 (35)</td>
<td>17 (36)</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>15.0</td>
<td>26.0</td>
<td>25.0</td>
</tr>
<tr>
<td>No. of abortions</td>
<td>0</td>
<td>1+</td>
<td>1</td>
</tr>
</tbody>
</table>

*ectopic pregnancy.
into the uterine cavity. Clinical pregnancies were defined by the presence of a gestational sac with fetal heartbeat. The implantation rate represents the ratio between the number of gestational sacs with fetal heartbeat and the total number of embryos transferred. Before the embryo transfer procedure, all patients were recommended to undergo, in case of pregnancy, conventional prenatal diagnosis in order to confirm and complete the results obtained by PGD.

Statistical analysis
Data were analysed by $\chi^2$ analysis applying the Yates’ correction, $2 \times 2$ contingency tables.

Results
A total of 346 two pronuclear zygotes from 51 cycles underwent polar body biopsy (Table I). A FISH diagnosis was achieved for 260 zygotes (75%) of which 77 were chromosomally normal (22%), whereas 183 (53%) exhibited chromosomal abnormalities which were not compatible with either implantation or a healthy pregnancy. For the remaining 86 zygotes, 49 could not be FISH diagnosed due to technical problems during fixation, whereas partial results (for some chromosomes or for one polar body only) were obtained in 37 zygotes. In 67 of the resulting embryos with a normal development, blastomere biopsy was performed in order to complete the diagnosis (Table II).

As represented in Table II, in 19 cycles the FISH diagnosis was based on the results from polar body analysis. In total, 108 pronuclear zygotes were analysed; 29 were classified as chromosomally normal (27%) and 69 as aneuploid (64%). In 10 zygotes, a diagnosis could not be made, but blastomere biopsy was not performed due to poor embryo development. Three pregnancies derived from the transfer of FISH normal embryos in 13 cycles with an implantation rate of 15.0%.

Both polar body and blastomere biopsy was performed in 32 cycles. Following polar body analysis on 238 pronuclear zygotes, 48 zygotes were diagnosed as euploid (20%), 114 as aneuploid (48%), whereas for 76 no diagnosis was achieved (32%). Sixty-seven morphologically normal embryos developed on day 3 from these non-diagnosed zygotes, and one blastomere was removed in order to achieve a diagnosis: 31 were euploid and 36 were chromosomally abnormal. The FISH diagnosis obtained by combining the results derived from polar body biopsy and blastomere biopsy accounted for 79 embryos with a normal chromosomal complement (33%), 150 carrying chromosomal abnormalities (63%) and nine with no result (4%). Embryo transfer was cancelled in three of the started PGD cycles (9%) as no euploid embryos with a normal morphology were diagnosed; an average of 1.7 ± 0.6 embryos were transferred to the remaining 29 cycles yielding 10 clinical pregnancies with an implantation rate of 26.0%. In 12 of the 29 transferred cycles, only embryos which underwent both polar body and blastomere biopsy were transferred, generating five pregnancies (four on term and one ectopic) and an implantation rate of 37.5% (six gestational sacs with fetal heartbeat out of 16 embryos transferred). In the remaining 17 transferred cycles, each patient was transferred with two embryos; one embryo had a polar body biopsy and the other had both polar body and blastomere biopsy; five term pregnancies were obtained, two with two gestational sacs each, with an implantation rate of 20.6% (seven gestational sacs with fetal heartbeat out of 34 embryos transferred).

In the 62 cycles undergoing blastomere biopsy, 376 day 3 embryos were FISH analysed, resulting in 117 euploid embryos (31%) and 258 chromosomally abnormal embryos (69%); in one embryo, a diagnosis was not obtained. Embryo transfer was performed in 47 cycles (76%) generating 17 clinical pregnancies and an implantation rate of 25.0%. Twelve pregnancies went to term, four are ongoing beyond 12 weeks gestation and one ended in spontaneous abortion.
The morphology of the analysed embryos was evaluated in relation to the cleavage rate at the observations performed at 40, 62 and 88 h post-insemination. The proportion of 4-cell embryos on day 2 and of 8-cell embryos on day 3 was similar in the three groups (polar body biopsy, polar body biopsy associated with blastomere biopsy, and blastomere biopsy), suggesting that the procedure of polar body biopsy has no effect on embryo cleavage (Figure 2). In addition, the proportion of development to morula on day 4 was not different between the three groups, implying that a combined biopsy procedure has the same effect on the cleavage rate as a single procedure.

Similarly, the percentage of fragments in the perivitelline space seemed not to be dependent on the biopsy procedures as indicated by comparable proportions of embryos at the 4-cell, 8-cell and morula stage with no fragments observed on day 2, 3 and 4, respectively, in the three groups (Figure 3).

**Discussion**

PGD represents nowadays the only alternative for couples at high reproductive risk to have a healthy child without fear of repeated spontaneous or therapeutic abortions. According to the latest reports on international data, PGD is no longer considered a research activity although an additional effort is still required to enhance the reliability and accuracy of genetic diagnosis on preimplantation embryos (Gianaroli et al., 2001, 2002; ESHRE PGD Consortium Steering Committee, 2002; Kuliev and Verlinsky, 2002).

There are two major limiting factors in PGD techniques: time and the number of cells available for analysis. The protocols currently applied rely on the genetic analysis performed on polar bodies or blastomeres obtained from preimplantation embryos in a time frame that is compatible with the length of culture in IVF. This requisite has the aim of avoiding embryo cryopreservation due to the low survival rate of thawed biopsied embryos (Joris et al., 1999; Magli et al., 1999). Recently published results on cryopreservation methods seem to indicate a better performance by introducing some technical modifications (Jericho et al., 2003), but additional data are needed before this method is valid for clinical application. On the other hand, the availability of only one cell for analysis imposes restrictions on the type of diagnosis that can be provided. In the case of chromosomal analysis, cytogenetic testing is performed on interphase nuclei by multicolour FISH. However, even after rehybridization of the same cell with different sets of probes, the number of studied chromosomes is limited and certainly far from complete karyotyping. Alternatives have been proposed to improve the quality of the results, including the removal of two cells per embryo (Sermon and Libaers, 1999), comparative genomic hybridization (Wells et al., 2002; Wilton et al., 2003), multiplex fluorescent PCR using polymorphic microsatellite markers (Findlay et al., 1998; Sherlock et al., 1998), second
polar body or blastomere nuclear conversion (Verlinsky and Evsikov, 1999; Willadsen et al., 1999) and blastocyst biopsy (McArthur et al., 2003). Comparative genomic hybridization and blastomere nuclear conversion are the only techniques that currently allow complete karyotyping from single cells. However, both methods are extremely laborious and hardly compatible with the laboratory framework for PGD. The results derived from studies based on the removal of two cells from each embryo do not provide information on the maintenance of embryo implantation potential in relation the reduction of the embryonic mass. On the other hand, the clinical application of blastocyst biopsy, for which even less time is available to perform a diagnosis, is very recent and needs to be verified by adding more data. Chromosomal disorders can also be tested by DNA fingerprinting using multiplex fluorescent PCR; at present, this is only feasible on a few chromosomes (Findlay et al., 1999), but it can be combined with the screening for single-gene diseases: as recently reported, the screening for Down’s syndrome has been performed in the same experiment designed for testing cystic fibrosis (Katz et al., 2002). The whole genome amplification that permits chromosomal analysis via comparative genomic hybridization has also been shown to provide enough DNA for concurrent testing of single-gene disorders (Wells et al., 1999).

The above considerations are clearly indicative of a search for a system aimed at maximizing the clinical advantage of applying PGD in reproductive medicine. In view of these considerations, the present study explored the possibility of using both polar bodies and one blastomere from the same embryo as the source of DNA. In order to verify whether any negative effect on implantation could derive from the application of the combined biopsy procedures, a retrospective analysis was performed on cycles where the two biopsy procedures were applied simultaneously. This mostly occurred in cycles which had been programmed to undergo chromosomal analysis on polar bodies: when absent or incomplete FISH diagnosis was obtained, a further attempt was made by biopsying one blastomere from the corresponding day 3 embryo, if its development was within the normal range. In our centre, the indication for aneuploidy screening on polar bodies is part of a programmed schedule for patients with a poor prognosis (Figure 4). The first approach is generally represented by FISH on blastomeres; in cases of no transfer due to FISH results or no pregnancy after repeated PGD for aneuploidy cycles, FISH on sperm is recommended to the male partner and, if the results are within the normal range, a cycle with polar body biopsy is the following step. This is done with the purpose of trying to allocate to either gametes or embryos the origin of aneuploidy. The effects of the combined biopsy procedures were evaluated by comparing the results obtained in cycles where this strategy was applied with those where either polar body or embryo biopsy was performed. The cycles included in each group had similar characteristics in terms of poor prognosis, including maternal age and mean number of previous IVF failures.

Apparently, there was no difference in the timing of cell division nor in the proportion of embryos with fragmentation between embryos generated after the combined biopsy procedures versus those which underwent polar body biopsy or blastomere biopsy (Figures 2 and 3). These results suggest that the type of biopsy does not affect embryo cleavage and morphology at least until day 4 (Magli et al., 2001b).

Accordingly, the implantation rate in the group of combined biopsy procedures was found to be in the normal range found in poor prognosis patients after PGD for aneuploidy on a polar body or blastomeres (Table II) (Gianaroli et al., 1999a, 2003b). In 12 cycles, out of the 29 transferred, a direct correlation between implantation and combined biopsy procedures could be established as the 16 embryos transferred had undergone both polar body and blastomere biopsy, yielding an implantation rate of 37.5% (6/16). This figure was comparable with that observed in the other group of 17 transfers (20.6%; 7/34) where two embryos were replaced in each patient; one embryo developed after polar body biopsy and the other after polar body and blastomere biopsy. As two twin pregnancies derived from this group, a direct correlation between implantation and combined biopsy procedures could be established for 18 transferred embryos yielding eight gestational sacs. In addition, no significant differences in implantation rates were detected in comparison with cycles after a single biopsy procedure, either polar body biopsy (15.0%) or blastomere biopsy (25%). More results are needed to evaluate whether the slight decrease in the implantation rate detected after polar body biopsy could be due to a non-detected paternal contribution to aneuploidy or to mitotic errors generated at fertilization or during the first embryo divisions (Gianaroli et al., 1999a, 2000; Magli et al., 2002). Another factor contributing to the lower implantation rate after polar body biopsy could reside in the fact that blastomere biopsy was only performed on regularly developing embryos. Therefore, chromosomally normal embryos with a poor development were already selected out of this group.

In conclusion, these findings suggest that the removal of a blastomere subsequent to polar body biopsy does not alter embryo viability in comparison with a single biopsy procedure. Although the combined biopsy procedure increases notably the laboratory workload compared with either type of biopsy alone, the main applications of this approach could be (i) an increase in the number of chromosomes analysed for patients requiring aneuploidy screening; and (ii) the possibility of performing aneuploidy screening in combination with PGD for single-gene disorders, especially in cases at risk of generating aneuploid embryos. This approach was followed for two couples undergoing PGD for cystic fibrosis, yielding one term pregnancy in a 38-year-old woman who had already undergone three unsuccessful PGD cycles. Although encouraging, these results are preliminary and need to be confirmed by additional data, but possibly represent another step towards the amplification of the usefulness of PGD.

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