Re-analysis of 166 embryos not transferred after PGS with advanced reproductive maternal age as indication

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BACKGROUND: In a randomized controlled study aiming to test the effectiveness of preimplantation genetic screening (PGS) in women of advanced maternal age, embryos diagnosed as chromosomally abnormal and those with no diagnosis were fixed for reanalysis. The aim of this study was to determine how well the chromosomal constitution of one biopsied blastomere reflects the status of the entire embryo.

METHODS: One hundred and seventy-three embryos diagnosed as chromosomally abnormal, 22 with no PGS result and four degenerated embryos originally diagnosed as normal were fixed and reanalysed by fluorescence in situ hybridization.

RESULTS: In total, 199 embryos were fixed, of which 166 were successfully reanalysed. One hundred and sixty embryos were found to be chromosomally abnormal; 48 of the reanalysed embryos with an initial diagnosis (149) had at least one cell with exactly the same chromosomal constitution shown in the first PGS analysis (34.2%). The reanalysis confirmed the initial overall chromosomally abnormal status of the embryo in 95.9% of the cases. Of all chromosomally abnormal embryos, 4.1% were diagnosed as false positive. The risk for false negative rate was at least 4.1%.

CONCLUSIONS: PGS seems to be a good method for selecting against chromosomally abnormal embryos but not for determining an embryo’s exact chromosomal constitution.

Key words: PGS / embryo / chromosome / FISH / aneuploidy

Introduction

In vitro fertilization has shown a generally increased success rate over the last years. However, women of high reproductive age (over 37) have both a lower implantation rate and a higher risk of miscarriage than younger women. This has been assumed to be attributable to factors associated with the ageing of the oocyte, which may lead to meiotic and mitotic errors. An increased rate of chromosomal abnormalities has been found in the embryos of older women (Munne et al., 1995; Dailey et al., 1996).

At present, the selection of embryos for transfer is based mainly on developmental and morphological criteria. Some observational studies with matched controls have indicated increased pregnancy rates, decreased spontaneous miscarriage rates or both after selection of embryos when preimplantation genetic screening (PGS) is performed (Gianaroli et al., 1999; Munné et al., 2003, 2007a, b). However, more recent prospective randomized controlled trials (RCTs) could not find positive effects of PGS (Staessen et al., 2004, 2008; Stevens et al., 2004; Debrock et al., 2007; Mastenbroek et al., 2007; Hardarson et al., 2008). Two of these trials (Mastenbroek et al., 2007; Hardarson et al., 2008) even showed a significantly lower pregnancy rate in patients undergoing PGS.

In an RCT for PGS at Reproductive Medicine, Sahlgrenska University Hospital and Fertility Centre, Carlanderska Hospital, Gothenburg, Sweden, 109 women 38 years of age and over were randomized either to embryo biopsy and PGS or to conventional morphological selection of the embryos. In the biopsy group (56 patients), one cell was biopsied from all good quality embryos on Day 3 and analysed by fluorescent in situ hybridization (FISH) for chromosomes 13, 16, 18, 21, 22, X and Y (Hardarson et al., 2008).

When available, one or two embryos with biopsied cells that were chromosomally normal were selected for transfer. For embryos with
biopsied cells that were chromosomally abnormal or for which no FISH result was obtained, all remaining cells were fixed for reanalysis by FISH. Here we report the results of 166 embryos reanalysed after PGS. The aim of this study was to determine how well the chromosomal constitution of one biopsied blastomere reflects the status of the entire embryo.

Materials and Methods

Pre-embryos

Out of 338 embryos eligible for biopsy, 332 went to FISH analysis; 302 (91%) were successfully analysed and 30 (9%) could not be analysed (Hardarson et al., 2008). In all, 204 of the analysed embryos were chromosomally abnormal and 173 (84.8%) of these were fixed for reanalysis. When embryos were not fixed it was due either to grave degeneration or to logistical reasons (e.g. lack of personnel). Of the embryos that could not originally be analysed mainly due to degeneration (i.e. no nucleus found), 22 were fixed for reanalysis. In addition, four arrested and morphologically degenerated embryos originally diagnosed as normal were fixed. In total 199 embryos were fixed for reanalysis (Fig. 1).

Fixation of interphase nuclei, FISH procedure

The embryos were fixed on glass slides using 0.01 N HCl + 0.1% Tween 20 as first introduced by Coonen et al. (1994). Care was taken to remove as much of the cytoplasm as possible from the nuclei to improve the FISH probe penetration.

The slides were washed for 1 min in 1× phosphate buffered saline (PBS) followed by an ethanol series of 70, 85 and 99.5% for 1 min each. For simultaneous detection of five chromosomes, we used a multicolour kit (MultiVysion PGT multi-colour Probe Panel, Vysis/Abbott Inc., USA), which includes chromosomes 13, 18, 21, X and Y. 0.3 μl of the probe was pipetted onto the nuclei, a cover glass applied and glued and the preparation denatured at 80 ± 1°C for 5 min on a heating plate. The slides were incubated at 38 ± 1°C overnight in a moist chamber; the cover glasses were then removed and the slides washed at 73 ± 1°C in 0.4× saline-sodium citrate (SSC) buffer (2 min) followed by 2× SSC+0.1% Nonidet P-40 (NP40) for 10–15 s to remove non-specific staining. The slides were thereafter air-dried, antifade solution applied and the preparation sealed with a cover glass. The nuclei were observed with an epifluorescence microscope equipped with appropriate filters. Before the second FISH round, the cover glass was carefully removed and the slides were then washed for 10 min in 1× PBS. After dehydration in ethanol series, a probe solution for chromosomes 16 and 22 was

Figure 1 Embryo flow diagram.
applied (Vysis/Abbott Inc., USA) using the same FISH procedure as described above.

**FISH scoring criteria**

FISH signals had to be at least of a signal’s width apart to be scored as two separate signals. If there was any doubt about the number of FISH signals, the aberrant alternative was chosen.

The embryos were categorized according to the following criteria:

(i) Overall normal: >50% normal diploid cells.
(ii) Aneuploid: same aberration found in 80% or more of the cells; no normal cells.
(iii) Aneuploid mosaic: same aberration in several (but fewer than 80%) cells in combination with other errors; some normal diploid cells commonly found.
(iv) Chaotic: different aberrations in different cells; no specific chromosome involved; all cells abnormal.
(v) Partially chaotic: different aberrations in most cells; no specific chromosome involved; some normal diploid cells found.
(vi) Haploid mosaic: a mainly haploid embryo with some diploid cells.
(vii) Polyploid mosaic: a mainly polyploid embryo with some diploid cells.

**Results**

Out of 199 embryos fixed for reanalysis, 166 (83.4%) embryos from 48 patients were successfully analysed (Fig. 1). One hundred and sixty embryos were found to be chromosomally abnormal including four arrested embryos originally diagnosed as normal at PGS analysis, which means that the real rate of false negatives could be higher than 4.1%. In two of these embryos all reanalysed cells were normal diploid, i.e. the cell biopsied during the PGS procedure was the only abnormal cell. All four arrested embryos diagnosed as normal at PGS analysis were shown to be chromosomally overall abnormal. All four of them showed mosaicism (three partially chaotic and one aneuploid mosaic). Two of the partially chaotic embryos had one normal diploid cell and the third had two normal diploid cells. The aneuploid mosaic embryo had three normal diploid cells. In the cases of the two partially chaotic embryos with one normal diploid cell only, it was the biopsied cell which was normal.

The reanalysis confirmed the initial overall (≥50%) chromosomally abnormal status of the embryo in 139 (95.9%) of the 145 abnormal embryos reanalysed. Fifty one (34.2%) of the 149 reanalysed (including the four normal embryos as analysed in the initial PGS analysis) embryos with a PGS diagnosis had one or more cells with exactly the same chromosome constitution as in the first PGS analysis (Table I). Only 16 (10.7%) of all the reanalysed embryos had exactly the same chromosomal aberration (i.e. in >50% of the cells) as found in the initial PGS analysis.

Seventy eight (52.3%) of all 149 reanalysed embryos (with a PGS diagnosis) were classified as either partially chaotic (40, 26.9%) or aneuploid mosaic (38, 25.5%) (Table II and Supplementary Data Appendix 1).

**Discussion**

In this study, a high overall abnormality rate was found in the reanalysed embryos, indicating that embryos with one abnormal blastomere have a high risk of hosting the same or other chromosomal abnormalities in the other cells. Thus, the PGS diagnostic seems to be successful in selecting against chromosomally abnormal embryos. However, overall accordance between the initial PGS analysis and the reanalysis was rather low (10.7%). As at least one or several normal cells were found in 54 (37.2%) of the aberrant embryos, it cannot be excluded that in some cases a normal blastomere could have been biopsied from an overall abnormal embryo during the PGS analysis. In fact, this seems to have happened in the four growth-arrested embryos diagnosed as normal at PGS analysis, which after reanalysis were shown to be abnormal. All four of them showed mosaicism. These four embryos represent 4.1% of the 98 embryos diagnosed as normal after PGS analysis, which means that the real rate of false negatives could be higher than 4.1%.

<table>
<thead>
<tr>
<th>Initial PGS diagnosis</th>
<th>Number of embryos</th>
<th>Normal/Abnormal confirmed by reanalysis</th>
<th>Overall exactly same diagnosis</th>
<th>Same diagnosis in one or more cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multinuclear</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Nullisomic Nulli-/monosomic</td>
<td>8</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Monosomic</td>
<td>53</td>
<td>51</td>
<td>6</td>
<td>21</td>
</tr>
<tr>
<td>Mono-/trisomic</td>
<td>14</td>
<td>14</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Haploid</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Polyploid Tetrploid</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Tetrasomic Tetra-/hexasomic</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Trisomic Tri-/tetrasomic</td>
<td>37</td>
<td>35</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>Normal</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Total (%)</td>
<td>149 (100)</td>
<td>139 (93.3)</td>
<td>16 (10.7)</td>
<td>51 (34.2)</td>
</tr>
</tbody>
</table>
Despite the high frequency of mosaic embryos, we found a low frequency of false positives (4.1%). Other studies have found false positive rates between 0.6 and 17% when reanalysing embryos after PGS [Mantzourtzou et al., 2007 (0.6%); Cooper et al., 2006 (3.8%); Li et al., 2005 (7.8%); Staessen et al., 2004 (8.4%); DeUgarte et al., 2008 (17%)]. In all of these studies, except DeUgarte et al., a classification of 85–90% normal diploid cells was used to define normal embryos. If the same classification had been used in our study none of our embryos would have been classified as normal, since all six of our ‘normal’ embryos harboured less than 88% normal cells. However, it is unclear what frequency of normal cells is needed for an embryo to develop normally. Thus, some of the false positive embryos in our study may not have been viable due to too few normal cells in the embryo. Taking together the number of false positive embryos (6) and false negative embryos (4) found, the overall frequency of misdiagnosis was 6.7%. Although the risk of misdiagnosis would be lower if two cells were analysed in each embryo, this has to be weighed against the possible detrimental effect on the embryo of removing two cells (Goossens et al., 2008).

It has been speculated that one of the reasons for the low success rate in PGS is due to mosaicism; a number of chromosomally normal embryos are diagnosed as abnormal. However, the observed frequency of false positive diagnosed embryos was low in our study, indicating that although mosaicism is common, the explanation for the low success rate cannot be completely accounted for by this group of embryos. As we only have genetic information in our study from 4 of 98 embryos diagnosed as normal, we cannot exclude that the rate of false negatives may be substantially higher than 4.1%. In the study by DeUgarte et al. made on embryos not transferred after PGD, 8 (19%) out of 43 reanalysed ‘normal’ embryos were found to be chromosomally abnormal (DeUgarte et al., 2008). Thus, we cannot exclude the possibility that mosaicism in embryos being diagnosed as normal and therefore transferred may influence the results negatively.

The main mechanism behind the two most common chromosomally aberrant groups; partially chaotic (26.9%) and aneuploid mosaics (25.5%), seems to be post-zygotic because the occurrence of normal diploid cells is common in these groups. In contrast the mechanism behind aneuploid (22.1%) and chaotic (16.8%) embryos is probably meiotic, since no normal diploid cells are found in these groups. The most common causes of aneuploid mosaics, according to Delhanty (2005), are chromosome loss, followed by chromosome gain and, less frequently, by mitotic non-disjunction; all may be related to maternal age. Chaotic embryos, on the other hand, may arise independently of maternal age, be related to centrosome anomalies, and hence be of male origin (Delhanty, 2005). Wilding et al. (2003) suggest a different theory concerning the origin of chaotics, postulating a relation between maternal age and the frequency of chaotic embryos. They assert that maternal age has a strong influence on oocyte quality, where accumulated damage to the mitochondria throughout the mother’s life results in lowered mitochondrial activity in the oocytes. They found a significant increase of chaotic embryos from women over 37 years of age, which could suggest that degeneration of the mitochondria leads to decreased mitochondrial activity, in turn resulting in abnormalities in the meiotic apparatus. The observation that the frequency in chaotic and partially chaotic embryos in our material is rather high (16.8 and 26.9%, respectively) may support the hypothesis that a chaotic chromosome constitution is more common in embryos of older women. The maternal age effect on embryos may also be indicated by the fact that a majority of the embryos in this study had a more complex aneuploidy, involving more chromosomes, compared with what we found in embryos from the ordinary IVF program (younger women) in two earlier studies (Hardarson et al., 2001, 2003).

In conclusion, PGS seems overall to be an effective method for selecting against chromosomally abnormal embryos, but is less reliable for determining the exact chromosomal constitution of an embryo. The risk for false positive diagnosis is 4.1%. The risk for false negative results could not be evaluated, but is at least 4.1%.

### Supplementary data

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### References
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| Table II Distribution of the 149 embryos with a PGS diagnosis into different categories based on chromosomal constitution (for more details see Supplementary Data Appendix 1) |
|---------------------------------|-----------------|
| No of embryos                  | %               |
| Aneuploid                      | 33              | 22.1            |
| Aneuploid mosaic               | 38              | 25.5            |
| Chaotic                        | 25              | 16.8            |
| Partially chaotic              | 40              | 26.9            |
| Haploid mosaic                 | 4               | 2.7             |
| Polyploid mosaic               | 3               | 2.0             |
| Overall normal                 | 6               | 4.0             |
| Total                          | 149             | 100             |


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