

Preimplantation Genetic Diagnosis and Human Implantation—A Review

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By selecting chromosomally normal embryos for replacement, PGD for aneuploidy can (i) increase implantation rates, (ii) reduce spontaneous abortion rates, and (iii) avoid aneuploid conceptions. When eight chromosomes are analysed, a significant increase in implantation is achieved. PGD is also found to significantly reduce the incidence of spontaneous abortion and chromosomally abnormal conceptions.

PGD for patients of advanced maternal age with an adequate number of embryos will improve their chances of childbirth via improved implantation and sustained gestation. PGD has also been used to help other specific groups of patients with high rates of chromosome abnormalities such as patients with recurrent spontaneous abortions, non-obstructive azoospermia, repeated IVF failure, and patients previous trisomic offspring.

Recently, PGD has been performed using comparative genome hybridization, which counts all chromosomes; but time constraints require embryo cryopreservation, which reduces the potential of improved implantation

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INTRODUCTION

The rationale for using Preimplantation Genetic Diagnosis (PGD) in order to increase implantation rates and reduce miscarriage rates is as follows. Oocyte quality is the major cause of reduced implantation with advancing maternal age (Navot et al., 1994) and the clearest link so far between maternal age and embryo competence is aneuploidy. Increased aneuploidy with maternal age in spontaneous abortuses and live offspring (Hassold et al., 1980; Warburton et al., 1980, 1986; Simpson, 1990) was also present in cleavage-stage embryos and oocytes (Munné et al., 1995a; Dailey et al., 1996; Márquez et al., 2000) but with much higher rates of chromosome abnormalities than in spontaneous abortions, strongly suggesting that a sizable part of chromosomally abnormal embryos are eliminated before clinical recognition. This embryo loss largely accounts for the decline in implantation with maternal age.

Because of the correlation between aneuploidy and declining implantation with maternal age, it was hypothesized that negative selection of chromosomally abnormal embryos could reverse this trend (Munné et al., 1993). Currently, negative selection of aneuploid embryos can only be done through PGD, by either polar body or blastomere analysis. Fluorescence in-situ hybridization (FISH) allows chromosome enumeration to be performed on interphase cell nuclei, i.e. without the need for culturing cells or preparing metaphase spreads. FISH has been used for PGD of common human aneuploidies with either blastomeres (cells from 2 to 16-cell

stage embryos) or oocyte polar bodies (Munné et al., 1993, 1995a,b, 1998a,b,c; Verlinsky et al., 1995, 1996, 1998a,b, 1999, 2001; Manor et al., 1996; Munné and Weier, 1996; Verlinsky and Kuliev, 1996; Gianaroli et al., 1997; Gianaroli et al., 1999, 2001b; Pehlivan et al., 2002). Currently, probes for chromosomes X, Y, 13, 14, 15, 16, 18, 21 and 22 are being used simultaneously (Bahçe et al., 2000), with the potential of detecting 70 per cent of the same aneuploidies found in spontaneous abortions. While the probes check only a limited number of chromosomes, the results so far indicate that PGD of aneuploidy actually does increase implantation rates while reducing trisomic offspring and spontaneous abortions (Munné et al., 1999, 2003; Gianaroli et al., 1999, 2001b; Gianaroli, Magli and Ferraretti, 2001a; Werlin et al., 2003). So far, more than 2000 cycles of PGD of aneuploidy have been reported by the three leading centres: RGI (Chicago, IL, USA); Saint Barnabas Medical Center (Livingston, NJ, USA); and SISMeR (Bologna, Italy) (International Working Group on Preimplantation Genetics, 2001).

Three centres that have performed the most PGD of aneuploidy have recently reported the birth of 515 babies after this procedure (Verlinsky et al., 2003).

RESULTS OF PGD FOR ANEUPLOIDY: TRISOMIC OFFSPRING, SPONTANEOUS ABORTIONS AND IMPLANTATION

Reduction in trisomic offspring

So far, more than 2000 cases of PGD of aneuploidy have been performed, either using embryo biopsy or polar body biopsy

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(Munné et al., 1999, 2003; Gianaroli et al., 1999, 2001b; Verlinsky and Kuliev, 2001). Large numbers are needed to demonstrate a decrease in trisomic offspring, from the 2.6 per cent trisomies for chromosomes 13, 18 or 21 detected in CVS in women 39 years old, to 0.3 per cent after PGD (assuming a 10 per cent error rate). Indeed, four misdiagnoses have already occurred after PGD (Munné et al., 1998c and this review with 2/334 fetuses misdiagnosed; Gianaroli, Magli and Ferraretti, 2001a, with 2/91 fetuses misdiagnosed). In all cases reanalysis of the misdiagnosed cells with probes binding to a different locus confirmed prior results, indicating that the errors were probably caused by mosaicism. This is in accordance with our recently published study on mosaicism, in which 7.2 per cent of embryos not replaced after PGD were shown to have been misdiagnosed, and of those 60 per cent of misdiagnoses were caused by mosaicism (Munné et al., 2002).

In spite of these misdiagnoses, the rate of trisomic offspring detected after PGD is lower than expected (Gianaroli, Magli and Ferraretti, 2001a). For instance, two of 314 (0.6 per cent) fetuses were found with aneuploidies for chromosomes XY, 13, 15, 16, 18, 21, and 22 (Munné, 2003) compared to a 2.6 per cent rate expected in a population of the same age range. That is a four-fold decrease of aneuploid conceptions after PGD.

If our data were to be combined with that of Verlinsky's group (Verlinsky et al., 2001), who reported 140 healthy children born after PGD of aneuploidy using polar body analysis, the overall reduction in trisomic offspring would be highly significant.

Decrease in spontaneous abortions

In a multicentre IVF study controls were compared with a test group undergoing embryo biopsy and preimplantation genetic diagnosis for aneuploidy of chromosomes X, Y, 13, 18 and 21 (Munné et al., 1999). The results revealed a significant decrease in spontaneous abortions (measured as the ratio of fetuses with heartbeats that aborted against the total number of fetal heartbeats detected) after PGD (from 23 per cent to 9 per cent, $P<0.05$) and ongoing pregnancies and delivered babies increased in the PGD group of patients (from 10.5 per cent to 16.1 per cent, $P<0.05$) (Munné et al., 1999).

Jobanputra et al. (2002) have recently reported that FISH with probes for 13, 15, 16, 18, 21, 22, X and Y can detect 83 per cent of all chromosomally abnormal fetuses detected by karyotyping. Since this combination of probes is the current standard (Munné et al., 1998a, 1999; Gianaroli et al., 1999), PGD should also eliminate close to 80 per cent of all chromosomally abnormal embryos at risk of causing a miscarriage. For instance, an abortion rate of only 9 per cent was reported after PGD of aneuploidy for 343 cases in women >36 years (Gianaroli, Magli and Ferraretti, 2001a; Gianaroli et al., 2001b).

Increase in implantation rates

When comparing the PGD patient group to a control group, each with average maternal age of 36 years and using probes

for the same chromosomes as our first study (Munné et al., 1999), plus probes for chromosomes 15, 16 and 22, we observed a significant two-fold increase of implantation, from 10.2 per cent to 22.5 per cent ($P<0.001$) (Gianaroli et al., 1999).

The low increase of implantation after embryo biopsy and PGD of aneuploidy in our first study (Munné et al., 1999) could be attributed to the probes employed. The chromosomes chosen for that study showed abnormalities that were compatible with further development and/or that have low impact on implantation. After analyzing 1600 embryos for different chromosomes, the most common aneuploidies found were those for chromosomes 22, 16, 15, and 21 while those for chromosomes XY, 14, 6, and 18 were the less common (reviewed by Munné, Sandalinas and Cohen, 2001, and unpublished data). Thus, when probes for chromosomes 16, 22 and 15 were added to the original protocol using XY, 13, 18, 21 probes (Munné et al., 1999), the improvement in implantation became significant (Gianaroli et al., 1999, 2001b; Munné et al., 2003).

In the latest study, using probes for X, Y, 13, 15, 16, 17, 18, 21 and 22 chromosomes, a group of patients with average maternal age of 40 years old and with two or less previously failed IVF attempts undergoing PGD were compared to a control group of the same characteristics. The PGD group showed a 20 per cent implantation rate compared to a 10 per cent rate in the control group ($P=0.002$) (Munné et al., 2003).

No reports on implantation rates comparing PGD and controls have been published so far regarding polar body analysis. Although this variant of PGD is in theory less prone to misdiagnosis due to mosaicism, other errors can occur that are particular to polar body analysis. In addition, post-zygotic abnormalities, which account for more than half of all chromosome abnormalities, cannot be detected.

Reduction of multiples and frozen embryos

PGD of aneuploidy may also help reduce the number of multiple pregnancies. In the two latest studies involving a test and a control group, significantly fewer embryos were transferred in the PGD groups than in control groups (Gianaroli et al., 1999; Munné et al., 1999, 2003; Werlin et al., 2003).

In addition, because many embryos are abnormal, after PGD there are fewer chromosomally normal embryos remaining in excess of those for replacement. Therefore there are very few embryos freezable, which will alleviate the problem of accumulating, storing and eventually disposing of unwanted frozen embryos.

INDICATIONS FOR PGD OF ANEUPLOIDY

Advanced maternal age

This group of patients has already been discussed before. Although the highest increase in aneuploidy detected at

cleavage stage occurs after age 39 (Munné et al., 1995a; Márquez et al., 2000), our most recent data indicates that implantation for patients 35–39 also may increase after PGD although the difference with controls is still not statistically significant (Munné et al., 2003).

Data from other centres indicates similar implantation for PGD cycles from 36 to 42; decreasing only when there are insufficient normal embryos for transfer (Gianaroli et al., 2001b). Despite the high rate of chromosome abnormalities after age 40, even single embryo transfers after PGD have resulted in acceptable implantation and pregnancy per transfer (35 per cent) in a group of patients with an average maternal age of 42 (Obasaju et al., 2001).

Recurrent miscarriages (RM)

Recurrent miscarriage (RM) in patients with normal karyotype is defined as three or more consecutive spontaneous abortions of less than 20–28 weeks' gestation (Stephenson, 1996). This is because, if 15 per cent of pregnancies spontaneously abort (Warburton and Frazer, 1964), the probability of three miscarriages occurring by chance should be 0.3 per cent instead of the observed 1 per cent in RM (Quenby et al., 2002).

Chromosome abnormalities are the major cause of miscarriage, with 99 per cent of chromosomally abnormal pregnancies miscarrying (Jacobs and Hassold, 1987) compared to 7 per cent chromosomally normal (McFadyen, 1989). However, the frequency of abnormal embryonic karyotypes has been found to be higher in sporadic abortions (63–76 per cent) than in RM (40–60 per cent) (Jacobs and Hassold, 1987; Stern, Dorfman and Gutierrez-Najar, 1996; Ogasawara et al., 2000; Stephenson, Awartani and Robinson, 2002). In addition, women aborting 2–4 consecutive pregnancies had 60 per cent chromosomally abnormal abortuses but women with >4 RM had only 29 per cent abnormal (Ogasawara et al., 2000).

All the previously mentioned studies were performed on products of conception of clinically recognized pregnancies. The IVI group of Valencia (Simon et al., 1998; Vidal et al., 1998; Pellicer et al., 1999; Rubio et al., 2003) have detected significantly more chromosome abnormalities in cleavage-stage embryos from women with RM than in their respective control groups (71 per cent vs 45 per cent, $P < 0.0001$) (Rubio et al., 2003). No differences in pregnancy or implantation were observed between these groups.

Repeated IVF failure (RIF)

RIF is usually defined as three or more failed IVF attempts or implantation failure after the replacement of more than 10 embryos. However this classification will select different patients in different IVF centres with different pregnancy rates. Several groups have published their results on PGD for the indication of repeated IVF failure (Gianaroli et al., 1997, 1999, 2001b; Kahraman et al., 2000; ESHRE PGD Consortium Steering Committee, 2002; Pehlivan et al., 2002).

According to Gianaroli et al. (1997), chromosome abnormalities increased with increasing number of failed cycles, from 40 per cent with 2, to 50 per cent with 3 to 67 per cent with 5 or more. In another study, they found higher rates of chromosome abnormalities (60 per cent) than in controls in a population of patients relatively young (av. mat. age 32), with the majority of the abnormalities not being aneuploidy but mosaicism, polyploidy and haploidy (Gianaroli et al., 1999, 2001b). A study by Pehlivan et al. (2002) also found more chromosome abnormalities in RIF (67 per cent, av. mat. age 36) than in controls (32 per cent, av. mat. age 31.6), but most of the abnormalities (75 per cent) were aneuploidy and not mosaics. Further differences were found by us, because in patients with ≥ 2 previous cycles we found only 31 per cent (av. mat. age 40) chromosome abnormalities, similar to the 33.4 per cent (av. mat. age 40) found in controls (Munné et al., 2003). The difference in maternal ages, and number of failed cycles in these may be the cause of the detected differences in types and frequencies of abnormalities.

Because of the high rates of abnormalities detected by several studies in RIF patients (Gianaroli et al., 1999, 2001b; Pehlivan et al., 2002), PGD has been suggested as a mean of improving the odds of conceiving in these patients. One of these groups (Gianaroli et al., 1999, 2001b) reported results on 66 PGD cycles of RIF (av. mat. age 32). Implantation for the PGD group (17 per cent) was not statistically different from the control group (10 per cent) (Gianaroli et al., 1999). A third study by the ESHRE PGD Consortium Steering Committee (2002), which covered cases from 25 centres, reported a pregnancy rate for RIF of only 7 per cent per retrieval, compared to 28 per cent for PGD of aneuploidy cases with the indication of advanced maternal age or recurrent miscarriages. A study by Pehlivan et al. (2002) showed an implantation rate of 19.8 per cent in the RIF group (av. mat. age 36.2) compared to a 24 per cent in a group of PGD for X-linked diseases (av. mat. age 32), but they were not compared to RIF patients that did not undergo PGD. Finally, in a prospective randomized study, still ongoing, preliminary results found that the PGD group had a pregnancy rate of 20 per cent (2/10) and the control of 0 per cent (0/9) (Werlin et al., 2003).

From these studies there is no clear indication that RIF patients are benefited from PGD using FISH. There may be other reasons for this lack of success and these patients may have other problems than chromosomally abnormal embryos. For instance, the limited experience on cytoplasm transfer on RIF patients indicates that they benefited from a higher implantation and pregnancy rate (Brenner et al., 2000; Barritt, Cohen and Brenner, 2000). In addition, several reports indicate that these patients may have high rates of structural chromosome abnormalities (Gekas et al., 2001; Raziell et al., 2002), which will not be detected by PGD of aneuploidy.

Non-obstructive azoospermia

Another group that could benefit from PGD are extreme male infertility cases requiring epididymal sperm aspiration

(MESA) or testicular sperm extraction (TESE) (Gianaroli et al., 2000, 2001b) because their embryos have high rates of chromosome abnormalities (Gianaroli et al., 2001b; Silber et al., 2003).

We recently compared PGD cycles of ICSI for oligospermia (control group) to PGD cycles of ICSI for non-obstructive azoospermia using TESE (TESE group) (Silber et al., 2003). Only female patients 28–39 years old were included to minimize the effect of maternal-age-related aneuploidy. Chromosome enumeration was performed by FISH using 5 to 8 probes. The control group produced 43 per cent normal, 26 per cent aneuploid and 26 per cent mosaic embryos. In contrast, the TESE group produced 24 per cent normal, 16 per cent aneuploid and 56 per cent mosaic embryos. The difference in mosaicism rate of control and TESE embryos was highly significant ($P < 0.001$). Most of the mosaic embryos from the TESE group were chaotic, with most or all cells being chromosomally abnormal but different from each other (thus mosaic). Because the male centrosome is the organizing centre for the first mitotic spindle, a sperm abnormality may produce mosaicism in subsequent embryos. TESE sperm (particularly in cases of non-obstructive azoospermia) may have a higher incidence of compromised or immature centrosome structures leading to mosaicism in the embryo. Because all or most cells of chaotic mosaic embryos were abnormal, one cell is enough to detect the abnormality, and therefore it is recommended that TESE patients undergo PGD for numerical chromosome abnormalities (Silber et al., 2003).

Gonosomal mosaicism

Gianaroli et al. (2001b) studied patients with chromosome mosaicism detected in peripheral blood under the assumption that there may also be mosaics in the germ line. The sample studied is small to determine if they have more chromosome abnormalities than other groups.

However, a recent study by Robinson, McFadden and Stephenson (2001) indicates that gonosomal mosaicism is an unlikely cause of recurrent aneuploidy. They studied the parental and meiotic origin of trisomic spontaneous abortions to determine whether there was an increased incidence of paternal errors, or of alleles not present in either parent, indicating gonadal mosaicism. They looked at spontaneous abortions from 54 individuals (mean maternal age 37.9, mean RM 3.7) with 0.9 mean live births, determining the origin of trisomy as reported in their previous work (Robinson et al., 1999). When this group was compared to a control group with only one prior trisomic spontaneous abortion, the overall recurrence of the same abnormality occurred as often as expected by chance alone. In addition, the types of abnormalities found are similar to those found in single spontaneous abortions, almost all trisomies (35/37) were of maternal origin, there was no increased recurrence of the same abnormality within a family and the mean maternal age was high, suggesting this was the main factor.

Babies born and obstetric outcome

The three largest PGD centres have recently reported the birth of 515 babies after PGD of aneuploidy (Verlinsky et al., 2003). There are two reports on obstetric outcome of babies born after IVF and PGD (Strom et al., 2000; ESHRE PGD Consortium Steering Committee, 2002). With respect to the presence of congenital malformations in babies born after PGD, a 4.9 per cent major malformation rate has been reported (ESHRE PGD Consortium Steering Committee, 2002), which is slightly higher than the 3.8 per cent rate of major malformations found in non-PGD IVF patients (Bonduelle et al., 2002). However, the ESHRE PGD Consortium Steering Committee (2002) data was not calculated per each PGD test and there is no specific figure for PGD of aneuploidy.

Our current data on babies born after PGD of aneuploidy indicates a 4 per cent (3/75) of major congenital abnormalities. This is similar to the data reported by the ESHRE PGD consortium.

TOWARDS A FULL CHROMOSOME COUNT

Ultimately, speedy and efficient analysis of all 24 chromosomes is our true aspiration, as some embryos diagnosed as normal are undoubtedly still abnormal for other aneuploidies of chromosomes not analysed in current protocols; always considering that the error level, delay or damage caused by the procedure is compensated by the extra information obtained.

One approach has been to obtain metaphase chromosomes from blastomeres after converting the cell to metaphase stage (Verlinsky and Evsikov, 1999; Willadsen et al., 1999) or from fresh polar bodies without further micromanipulation since immediately after retrieval they are still at metaphase stage (Munné et al., 1998b). Metaphases can be analysed by regular karyotyping or Spectral Karyotyping Imaging (SKY) (Márquez, Cohen and Munné, 1998). However the rate of conversion is not perfect and the SKY method requires perfectly spread metaphases without overlaps.

Another approach has been to use molecular techniques to perform a quantitative analysis of the whole genome using either comparative genome hybridization (CGH) (Kallioniemi et al., 1992; Wells et al., 1999), with DNA fingerprinting (Findlay et al., 1998) or quantitative fluorescence multiplex PCR (QF-PCR) (Mansfield, 1993; Sherlock et al., 1998).

So far the most promising method is CGH, which can accurately determine total or partial aneusomy by loss or gains of DNA, using a combination of PCR and FISH technology. For CGH of single cells, the whole genome of the cell must be amplified beforehand (Wells et al., 1999). Trials applied to human blastomeres from discarded embryos have promising results (Voullaire et al., 1999, 2000; Wells and Delhanty 2000), but so far, the process takes too long. To gain enough time for analysis, Wilton et al. (2001) applied this method to blastomeres from embryos that were frozen after biopsy and

the first babies have recently been borne following this procedure. However, in our opinion, cryopreservation and thaw destroys some embryos and ultimately outweigh the benefits of CGH.

Recently, we have been able to obviate cryopreservation by applying CGH to polar bodies and get results prior to embryo replacement on day four of development (Wells et al., 2002). However, as with any polar body analysis, postzygotic abnormalities, which account for more than half all abnormalities, as well as paternally derived aneuploidies are not detectable.

Finally, DNA microarrays are being developed for aneuploidy and translocation analysis (Weier et al., 2001). The basis of this technique is similar to CGH with the difference that the substrate is not a metaphase but dots of probes on a glass chip, each dot representing a specific sequence of the genome. Thus, instead of hybridizing to metaphase chromosomes, the targets of hybridization are defined DNA sequences (for example centromeric and telomeric regions) attached to the slide.

CONCLUSION

PGD of aneuploidy has been shown to reduce the risk of trisomic offspring, increase implantation rates, and decrease spontaneous abortions. For the purpose of increasing implantation, PGD is a selection tool to screen from a cohort of embryos those that are chromosomally normal. However, it should only be applied when there are more embryos available for analysis than the number of embryos to be replaced.

PGD with FISH is an invasive method that may cause some damage to the embryo while providing an incomplete chromosome count. For the patients discussed above this damage is usually more than compensated by the advantages of screening for chromosome abnormalities, and when eight chromosomes are analysed, a significant implantation increase can be achieved.

The attempts at whole chromosome count through CGH and embryo freezing will probably increase both the damage to the embryo and the selection advantage, thus it remains to be seen if it compensates the effort. Faster and more robust techniques, such microarrays may obviate the need of embryo freezing and unleash the full potential of PGD.

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