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Current value of preimplantation genetic aneuploidy screening in IVF

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Preimplantation genetic aneuploidy screening (PGS) has been performed during the last decade as a way of enhancing embryo selection in patients with an increased incidence of embryonic numerical chromosome abnormalities (advanced maternal age, recurrent miscarriage and recurrent implantation failure). It has been proposed that the replacement of euploid embryos in these patients would result in a higher implantation and pregnancy rate and a reduced miscarriage rate. Additionally, the transfer of fewer embryos could reduce the chances for multiple pregnancies in all IVF patients. Although, to date, multiple studies have addressed this issue, contradictory results have been encountered. As a result, the effectiveness of aneuploidy screening remains to be established. Moreover, child outcome studies documenting the safety of this procedure are needed. The aim of this review is to summarize the available evidence concerning the use of PGS to determine the current value of the technique.

Key words: aneuploidy screening/embryo selection/preimplantation genetic diagnosis

Introduction

Since the beginning of IVF, many efforts have been made to enhance success rates, the optimization of embryo selection being one of the most evaluated strategies (Blake *et al*., 2005; Senn *et al*., 2006). Extensive evidence (Gianaroli *et al*., 1997, 1999; Staessen *et al*., 2004) has revealed a high incidence of embryo numerical chromosomal abnormalities (60–70%) in patients with a poor outcome after IVF (advanced maternal age, repeated IVF failure) as well as in the cases of unexplained recurrent miscarriage. Moreover, this has been observed despite good embryo quality, which offers a possible explanation for their low implantation potential. It has been reported that, in women over 37 years, only 35% of day 3 embryos with more than eight cells and 65% of expanding blastocysts are normal (Staessen *et al*., 2004). Hence, although embryo selection based on morphological evaluation either on day 3 or day 5 of development (percentage of cell fragmentation, number and size of the blastomeres and the presence of multinucleation) has shown to be effective (Kolibianakis *et al*., 2004; Papanikolaou *et al*., 2005), this approach could be inappropriate for these patients as it does not ensure a normal chromosomal constitution (Staessen *et al*., 2004; Li *et al*., 2005), resulting in implantation failure or miscarriage.

Preimplantation genetic aneuploidy screening (PGS) enables the assessment of the numerical chromosomal constitution of cleavage stage embryos through the use of fluorescence in-situ hybridization (FISH). Theoretically, the selection of euploid embryos for transfer would result in a higher implantation and pregnancy rate and a reduced miscarriage rate. In addition, fewer embryos could be transferred resulting in reduced chances for multiple pregnancies.

The first report of aneuploidy screening performed on a single cell in human embryos by multiple FISH probes (X, Y, 13, 18, 21) was conducted by Munné in 1993 (Munné *et al*., 1993).

To date, many studies have addressed the impact of PGS in different groups of patients; yet, its effectiveness has not been consistently proven as shown by a recent comprehensive review including both observational and randomized studies (Shahine and Cedars, 2006). Despite this, according to the European Society of Human Reproduction and Embryology (ESHRE) preimplantation genetic diagnosis (PGD) Consortium data collection, the number of PGD cycles performed for aneuploidy screening has increased considerably (1990 cycles from 1997 to 2001 compared with 1211 cycles in 2002), even though the overall reported clinical pregnancy rate per oocyte retrieval is only 16% (range: 12–33%) (Harper *et al*., 2006). Further data are required to establish whether PGS results in enhanced live birth rate, and if this is the case, to identify which patients may benefit. Additional points of interest for future research are (i) which chromosomes should be evaluated (as well as the added value of using more probes), (ii) the significance of mosaicism in the accuracy of PGS, the natural course of mosaicism and the advantages and disadvantages of

performing PGS one or two blastomeres removed, (iii) the implementation of new technologies for chromosome analysis such as comparative genomic hybridization (CGH) and (iv) the safety of this procedure evaluated through the follow-up of children born after PGS. This will only be accomplished through well-designed prospective (randomized) trials.

The aim of this review is to summarize currently available literature concerning the clinical value of PGS and to assess the intrinsic technical pitfalls linked to the technique itself, on the basis of evidence-based medicine principles.

Search strategy

A computer-based search was conducted through the bibliographic databases of Medline, Embase and Cochrane Menstrual Disorders and Subfertility group using the following key words: PGD, preimplantation genetic screening, aneuploidy screening, FISH, advanced maternal age, recurrent miscarriage and recurrent or repeated implantation failure. There was no language restriction.

How is PGS performed?

The technique for aneuploidy screening does not basically differ from PGD performed for inherited disorders. An ovarian stimulation protocol is followed by oocyte retrieval. As the method of insemination (ICSI or IVF) does not affect the effectiveness of the procedure, it can be selected as for non-PGD cycles (Thornhill *et al*., 2005).

In most centres practising PGS, the most frequent approach is the extraction of one or two blastomeres from a day 3 embryo, as at this stage, cells are thought to be totipotent and the embryo has not yet undergone compactation. The main advantage of studying two blastomeres instead of one is the achievement of improved diagnosis reliability as mosaic embryos can be identified (Baart *et al*., 2006). Concerns, however, have been raised regarding the safety of this strategy as it might interfere with the process of cell polarization (trophectoderm and inner cell mass) and cell differentiation. According to retrospective data, the extraction of two blastomeres instead of one does not impair either implantation or pregnancy rates (Van de Velde *et al*., 2000). A recent prospective randomized trial has also shown that there is no statistically significant difference in embryo development up to the blastocyst stage after PGS in case one or two cells are removed (46 versus 49%, respectively) (Goossens *et al*., 2005).

Only embryos with <50% of anucleate fragments are selected for biopsy. A hole is drilled within the zona pellucida using either acid Tyrode's or laser. Chemical drilling currently represents the most frequently used approach (Harper *et al*., 2006). Although both techniques result in comparable pregnancy rates, laser offers the advantages of being less time consuming and possibly yielding a higher rate of intact blastomeres (Joris *et al*., 2003).

Two other biopsy techniques are available: first and second polar body and blastocyst. Polar body biopsy is based on the fact that most aneuploidies derive from errors occurring during the first meiotic division of the oocyte. It has been shown, however, that to accurately predict the chromosomal constitution of the zygotes, the second polar body needs to be evaluated (II meiotic division) (Verlinsky *et al*., 2001; Kuliev and Verlinsky, 2004). The main advantage of polar body analysis is that it does not interfere with embryo development as polar bodies do not play a role in this process. Major disadvantages are the lack of evaluation of the paternal inherited genome and the fact that polar body analysis does not diagnose disorders arising during early embryo development.

The evaluation of trophectoderm cells from human blastocysts is a recently developed technique that enables multiple cells to be studied (range 2–9), thereby improving the likelihood of detecting mosaicism (McArthur *et al*., 2005). One additional advantage seems to be a higher rate of embryo survival (>90%) after the thawing of blastocysts compared with embryos biopsied on day 3 (Henman *et al*., 2005). The first live births after the transfer of biopsied blastocysts have already been reported (De Boer *et al*., 2002). Further studies are required to establish the future of this technique.

FISH procedure

FISH enables the numerical evaluation of chromosomes in interphase nuclei, thereby avoiding the performance of metaphase spreads. Different probes labelled with coloured fluorochromes for specific DNA detection are applied to the nuclear content of the blastomeres. After hybridization, each chromosome is identified and evaluated (Figure 1).

The selection of probes is based on the incidence of chromosomal abnormalities in spontaneous abortions and live births. It has been shown that using probes for chromosomes X, Y, 13, 16, 18, 21 and 22 enables the detection of 72% of the chromosomal abnormalities found in spontaneous abortions (Simpson and Bombard, 1987). To increase the number of analysed chromosomes, the same nucleus can be hybridized again with more probes; however, further hybridization reactions are likely to significantly reduce the test accuracy (Liu *et al*., 1998). Another strategy is to combine two or more fluorescent labels enabling the efficient assessment of 10 chromosomes within two hybridization reactions (Baart *et al*., 2004a).

A variable incidence of numerical chromosomal abnormalities has been reported (15–85%) depending on the studied population, number and type of used probes, as well as the quality of analysed embryos and the number of evaluated blastomeres (Bielanska *et al*., 2002). Table I summarizes the available evidence regarding the reported abnormality rates according to the studied population, number of biopsied cells and employed probes. The systematic analysis of preimplantation embryos has led to the conclusion that mitotic errors resulting in mosaicism are the most frequently found anomalies (~50%), followed by meiotic errors that generate consistent aneuploidy, where all blastomeres are affected (7.5–15%) (Trussler *et al*., 2004).

After FISH analysis has been performed, euploid embryos are selected for replacement on day 4 or 5 of development. Table II summarizes the scoring criteria according to FISH results.

Which patients can benefit from PGS?

The existence of a negative selection towards aneuploid embryos has been widely demonstrated in both *in vivo* and *in vitro* conditions, resulting in a low incidence of numerical chromosome abnormalities at birth (0.6%) (Hassold *et al*., 1980; Guerneri *et al*., 1987). In fact, it has been estimated that 70% of pregnancy losses occurring before 6 weeks of gestation are because of numerical chromosomal errors (Edmonds *et al*., 1982; Wilcox *et al*., 1988).

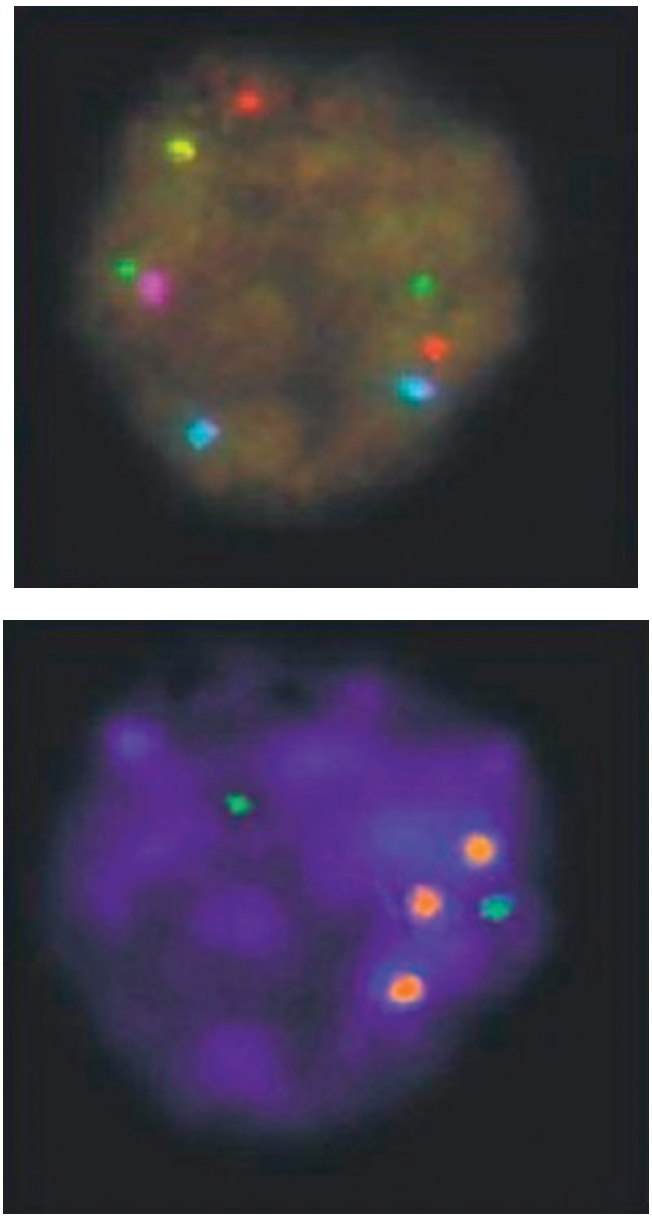

Figure 1. (**TOP**) A blastomere nucleus hybridized with probes specific for chromosomes 13 (red), 18 (aqua), 21 (green) X (purple) and Y (gold); the signal pattern is consistent with normal male chromosome complement. (**BOTTOM**) The same nucleus re-hybridized with probes specific for chromosomes 16 (orange) and 22 (green) and counterstained with 4,6–diamino-2-phenyl-indole (DAPI); the signal pattern is consistent with a normal complement for chromosome 22 and trisomy for chromosome 16.

After 10 weeks of pregnancy, however, miscarriage rate is reduced to 2–3% with chromosomal abnormalities being the cause of only 5% of these miscarriages (Simpson, 1990).

The recognition of a high aneuploidy rate in embryos from patients with advanced maternal age has led to an increased optimism with respect to the therapeutic potential of cytogenetic preimplantation embryo analysis. However, the recent report on a similar rate of euploid embryos (36%) for women <38 years than for older women, after performing FISH with 10 probes (Baart *et al*., 2006) has raised serious questions as to the value of aneuploidy screening in advanced maternal age women. In view of these results, a randomized trial including patients of all ages is mandatory. Additionally, an equal limited number of embryos should be replaced in both groups, especially in young women in which ideally single embryo transfer could be performed. To facilitate the comparison of data, it is also important to forge consensus on the definition of advanced maternal age because in currently available studies, it fluctuates between 35 and 38 years (Harper *et al*., 2006).

PGS has also been performed in patients with unexplained recurrent miscarriage, recurrent implantation failure, non-obstructive and obstructive azoospermia (NOA and OA) and severe sperm morphology anomalies. The evidence concerning these indications is analysed separately, given the existence of significant differences between them.

Advanced maternal age

As part of the lifestyle developed in Western societies, women frequently decide to delay child bearing, which results in an increased incidence of age-related fertility problems. The consistent finding of a high aneuploidy rate in embryos derived from older women (40–80%) (Gianaroli *et al*., 1997, 1999; Staessen *et al*., 2004; Platteau *et al*., 2005a), regardless of embryo morphology (Staessen *et al*., 2004), provided an explanation for their low implantation and high miscarriage rates after IVF treatment. Therefore, it was suggested that embryo selection based on the exclusion of numerical chromosomal abnormalities could improve the ongoing pregnancy rate and decrease the probability of trisomic offspring in these patients. Fewer embryos could thus be transferred with the additional benefit of reduced multiple pregnancy rates.

To date, most conducted studies have been descriptive (Platteau *et al*., 2005a) or observational, some of which have found a beneficial effect on implantation or pregnancy rates (Gianaroli *et al*., 1999; Munné *et al*., 1999, 2003). In one of the earliest trials, a significantly higher implantation rate following PGS compared with assisted hatching (25.8 versus 14.3%) was reported in a series of 157 patients >36 years who themselves chose between these two techniques. (Gianaroli *et al*., 1999). A multicentre retrospective study with a matched control group including 117 women >35 years observed no implantation rate improvement but described a significant increase in ongoing and delivered babies after PGS (16.1 versus 10.5%) (Munné *et al*., 1999). In addition, a larger series of 341 PGS cycles performed in women >38 years described an acceptable ongoing pregnancy rate per embryo transfer (28.8%) (Rubio *et al*., 2005). Although the authors presented a comparison with a group of women of the same age in whom PGS was not performed, no details regarding number and characteristics of these patients were given and no statistical analysis was reported.

Recently, a meta-analysis (Twisk *et al*., 2006) including two randomized studies (Staessen *et al*., 2004; Stevens *et al*., 2004) reported no difference in live birth rate [11% in the PGS group versus 15% in controls; odds ratio (OR) 0.65; 95% confidence interval (CI) 0.36–1.19], ongoing pregnancy rate per woman (15% in the PGS group versus 20% in controls; OR 0.64; 95% CI 0.37–1.09) and clinical pregnancy rate (15% in the PGS group versus 22% in controls; OR 0.42; 95% CI 0.12–1.51). This meta-analysis did not analyse implantation rates.

The largest randomized trial published to date (Staessen *et al*., 2004) analysed 289 cycles with oocyte retrieval from 400 patients

Table I. Chromosomal abnormality rates according to included patients, number of cells biopsied and FISH probes

Study	Patients	No of cells biopsied	Chromosomes tested using FISH	Abnormality rate (%)
Gianaroli *et al*. (1997)	AMA (≥38 years) (*n* = 11); RIF (≥2) (*n* = 22)	Not stated	X, Y, 13, 18, 21	AMA 63; RIF 57
Gianaroli *et al*. (1999)	AMA (≥36 years) (*n* = 157); RIF (≥3) (*n* = 54)	1	Six probes X, Y, 13, 16, 18, 21 Nine probes plus second hybridization 14, 15, 22	AMA: Six probes 64; Nine probes 64 RIF: 57; 45
Pellicer *et al*. (1999)	RM ≤36 years (*n* = 9) Control PGD sex-linked disease: ≤36 years (*n* = 10); >36 years (*n* = 9)	1 or 2	First hybridization X, Y, 18 Second hybridization 13, 21 Third hybridization 16, 22	RM 58.5 Control ≤36 years: 16.7; >36 years: 33.3
Kahraman *et al*. (2000)	AMA (mean age 37±2.1) (*n* = 49); RIF (≥2) (*n* = 23)	1	X, Y, 13, 18, 21	AMA 39; RIF 49
Pehlivan *et al*. (2003)	RIF (≥3) <37 years (*n* = 27) ≥37 years (*n* = 22) Control PGD sex-linked disease <37 years (*n* = 9)	1 or 2	First hybridization 13, 21 Second hybridization 16, 22 Third hybridization X, Y, 18	<37: 65.4; >37: 70.7; Control: 36.3
Munné *et al*. (2003)	AMA (≥35 years) (*n* = 138)	1	X, Y, 13, 15, 16, 18, 21 and 22	70.3
Abdelhadi *et al*. (2003)	426 embryos from women ≥35 years, RM, RIF or previous trisomic conception	1	First hybridization 13, 16, 18, 21, 22 Second hybridization X, Y, 15, 17 Third hybridization 2, 3, 4, 11	77
Rubio *et al*. (2003)	RM (≥2) <37 years (*n* = 51) ≥ 37 years (*n* = 20) Control PGD sex-linked disease <37 years (*n* = 15) ≥37 years (*n* = 13)	1 or 2	First hybridization 13, 21 Second hybridization 16, 22 Third hybridization X, Y, 18	<37: 70.7; 55.6 >37: 70.7; 59.3 Control: 45.1; 33.9
Werlin *et al*. (2003)	AMA (>38 years) (*n* = 19) RM (≥2) (*n* = 19) RIF (>2) (*n* = 19)	Not stated	13, 15. 16, 18, 18 ,21, 22, X, Y	AMA 53.7; RM 68.2; RIF 67.9
Staessen *et al*. (2004)	AMA (≥37 years) (*n* = 141)	2	First hybridization X, Y, 13, 18, 21 Second hybridization 16, 22	63.2
Platteau *et al*. (2004)	Non-obstructive azoospermia (*n* = 39 cycles) Obstructive azoospermia (*n* = 23 cycles) Control PGD sex-linked disease (*n* = 14)	2	First hybridization X, Y, 13, 18, 21 Second hybridization 16, 22	NOA: 52.5; 21 OA: 60; 30 Control: 40.5; 23
Kahraman *et al*. (2004)	Macrocephalic spermatozoa (*n* = 73) Absolute teratozoospermia (*n* = 71)	Not stated	13, 18, 21, X, Y	Macrocephalic spermatozoa 84.4, absolute teratozoospermia 93.3
Platteau *et al*. (2005)	AMA (≥37 years) (*n* = 279)	2	First hybridization X, Y, 13, 18, 21; Second hybridization 16, 22	65.3
Platteau *et al*. (2005)	RM (≥3) <37 (*n* = 35) ≥37 (*n* = 34)	2	First hybridization X, Y, 13, 18, 21 Second hybridization 16, 22	<37 years: 43.8; 39.9 ≥37 years: 66.9; 47
Munné *et al*. (2005)	RM (≥3) <35 (*n* = 21) ≥35 (*n* = 37)	1	First hybridization X, Y, 13, 16, 18, 21 Second hybridization 14, 15, 22	<35 years: 57 ≥years: 67
Baart *et al*. (2006)	Age <38 years (*n* = 60)	1 or 2	First hybridization 1, 7, 15, X, Y Second hybridization 16, 18, 13, ,21, 22	14

AMA, advanced maternal age; FISH, fluorescence in-situ hybridization; PGD, preimplantation genetic diagnosis; RIF, recurrent implantation failure; RM, recurrent miscarriage.

randomized at the out-patient clinic, evaluating seven chromosomes (X, Y, 13, 16, 18, 21 and 22) in two blastomeres. There was no significant increase in either the implantation (17.1% PGS versus 11.5% control) or the ongoing implantation rates (16.5% PGS versus 10.4% control). However, a higher number of embryos were replaced in the control group (2.8 versus 2), which render results of this study difficult to interpret. An increased number of transferred embryos in the control group could explain the comparable pregnancy rate per cycle achieved in this study. As regards the miscarriage rate, at least two observational studies have pointed to an increased ongoing pregnancy rate after PGS (Munné *et al*., 1999; Platteau *et al*., 2005a); however, the combined results

Table II. Scoring criteria according to FISH results when two blastomeres are evaluated

Euploid	Both blastomeres have two copies of each analysed chromosome
Aneuploid	Both blastomere nuclei have an abnormal signal copy number for the same chromosome
Haploid or Polyploid	Both blastomeres have one, three or more copies of every chromosome
Mosaic	One blastomere is euploid, the second blastomere has one chromosome with an abnormal number of copies or both blastomeres have different chromosomes with an abnormal copy number
Complex abnormalities	At least one blastomere has more than one chromosome with an abnormal number of copies

FISH, fluorescence in-situ hybridization.

of two randomized studies showed no significant difference (OR 0.27; 95% CI 0.04–1.82) (Twisk *et al*., 2006).

It has been suggested that not all advanced maternal age women may benefit from PGS. In fact, one retrospective study with a matched control group observed that, to increase implantation rates, there should be at least eight zygotes available and no more than two previously failed IVF treatments (19.2% PGS versus 8.8% control) (Munné *et al*., 2003). Other studies found that the most important prognostic factor is the presence of at least one chromosomally normal embryo (Ferraretti *et al*., 2004; Platteau *et al*., 2005a), because unchanged implantation rates (~10%) have been reported despite increasing age (37–43 years) (Platteau *et al*., 2005a). On the contrary, a poor prognosis has been observed when chromosomally abnormal embryos only are encountered, given that over 90% of these women repeat this result in a subsequent treatment cycle (Ferraretti *et al*., 2004).

Even though a trend towards a lower multiple pregnancy rate has been reported in observational studies (Oter *et al*., 2004; Platteau *et al*., 2005a) because of the transfer of fewer embryos following PGS, no significant differences were found in other studies (OR 0.41; 95% CI 0.12–1.36) (Staessen *et al*., 2004). Studies specifically designed to address the issue of multiple pregnancies are still awaited.

Recurrent miscarriage

Recurrent miscarriage is diagnosed when three consecutive pregnancy losses occur and has a prevalence of 1% (Li *et al*., 2002). In almost 50% of the cases, no cause is found (Li *et al*., 2002). Although the prognosis of establishing an ongoing pregnancy without further treatment in these patients is good (~70% live birth rate) (Clifford *et al*., 1997; Brigham *et al*., 1999), multiple therapeutic strategies have been proposed. The benefit of performing IVF (without a genetic assessment of embryos) remains controversial, because the two studies that evaluated this approach showed contradictory results (Balasch *et al*., 1996; Raziel *et al*., 1997).

The observation that spontaneous miscarriage is often because of the presence of *de novo* autosomal trisomies (13, 14, 15, 16, 21 and 22) (Hassold *et al*., 1980; Strom *et al*., 1992), coupled with the finding of a high incidence (50–60%) of numerical chromosomal abnormalities in embryos from couples with unexplained recurrent miscarriages (Pellicer *et al*., 1999; Rubio *et al*., 2003; Munné *et al*., 2005; Platteau *et al*., 2005b), led to the suggestion that IVF with PGS may be beneficial in these patients. To date, conflicting results have been reported, possibly because of heterogeneous inclusion criteria (two or three miscarriages and age of the women). In a series of 241 cycles, a similar outcome was observed after PGS compared with a control group of PGD cycles performed for sex-linked diseases, with a miscarriage rate of 12.3 and 8.3%, respectively (Rubio *et al*., 2005). The main criticism on this study is the use of an inappropriate control group, because these women did not have recurrent miscarriage. In another study, women >35 years of age showed a significant reduction in the miscarriage rate compared with the expected probability of miscarriage (estimated by age and number of previous abortions) (12 versus 45%, respectively) (Munné *et al*., 2005). Nevertheless, a comparison with a historical control group represents an important bias as with this methodology most interventions can be proven to be efficient, because these women already have a good chance to achieve a live birth without any intervention (Mastenbroek *et al*., 2006). On the contrary, Platteau *et al*. (2005b) reported no benefit of PGS in women >37 years (2.7% implantation rate and 5% ongoing pregnancy per transfer). The most probable cause for this poor result is that most of these older women also had infertility problems and consequently had significantly more chromosomally abnormal embryos than patients <37 years (66.9 versus 43.8%, respectively). The only randomized trial including couples with recurrent pregnancy loss (19 patients; 11 PGS and eight control) suggested an improved outcome after performing PGS (pregnancy rate per embryo transfer 63.6% PGS versus 37.5% control) (Werlin *et al*., 2003). Unfortunately, miscarriage rates were not reported. Moreover, the limited number of patients does not allow definitive conclusions to be drawn. Table III summarizes the results of these studies.

Hence, given the lack of evidence supporting a beneficial effect of PGS on live birth rate, this technique should not be performed on a routine basis. Only future well-designed randomized trials will establish the usefulness of PGS in recurrent miscarriage couples.

Recurrent implantation failure

Implantation is an extremely complex process that requires multiple factors to be synchronized: embryo quality, endometrial receptivity and the immune system. Recurrent implantation failure has been defined as three or more unsuccessful IVF cycles or the failure of conception after the replacement of 10 or more good quality embryos (ESHRE PGD Consortium Steering Committee, 2002). Multiple aetiologies have been proposed: increased incidence of numerical chromosomal abnormalities, disturbed endometrial receptivity, uterine pathology or an inadequate transfer technique.

The chance of success after three failed attempts depends on the age of the women, the number of oocytes retrieved and the quality of the embryos previously transferred. In older patients, a diminished oocyte quality because of cytoplasmic dysfunction may cause malsegregation of the chromosomes, thereby increasing aneuploidy rates, reducing chances for embryo implantation. Several studies have revealed a high aneuploidy rate in young women as well (Kahraman *et al*., 2000; Pehlivan *et al*., 2003). A correlation

Table III. Outcome of patients with unexplained recurrent miscarriage after performing PGS

Study	Study group	Control group	Outcome
Pellicer *et al*. (1999)	Nine patients ≤36 years three to six miscarriages	PGD sex-linked disease ≤36 years 10 patients >36 years six patients	Study group: three pregnancies one miscarriage Control group ≤36 years: three pregnancies one miscarriage >36 years: no pregnancies
Rubio *et al*. (2003)	51 patients <37 years, 20 patients ≥37 years	PGD sex-linked disease <37 years 15 patients ≥37 years 13 patients	Study group: miscarriage rate <37 years, 10.5% ≥37 years, 25% Control group: no miscarriage
Werlin *et al*. (2003)	11 patients ≥two miscarriages randomized for PGS	Eight patients ≥two miscarriages randomized for no PGS	PGS group: pregnancy rate/cycle 63.6% Control group: pregnancy rate/cycle 37.5%
Munné *et al*. (2005)	21 patients < 35 years, 37 patients ≥35 years	Same patients on cycle before PGS. Estimate of expected pregnancy loss according to Brigham *et al*. (1999)	Miscarriage rate <35 years: previous 90%, expected 29%, observed after PGS 23% Miscarriage rate ≤35 years: previous 86%, expected 44.5%, observed after PGS 12%
Platteau *et al*. (2005b)	35 cycles <37 years, 34 cycles ≥37 years	No	Ongoing pregnancy rate/oocyte retrieval <37 years 25.7% ≥37 years 2.9%
Rubio *et al*. (2005)	163 cycles <37 years, 78 cycles ≥37 years	25 cycles PGD for sex-linked diseases	Study group: miscarriage rate <37 years 10 ≥37 years 20 Control group: miscarriage rate 8.3%

PGD, preimplantation genetic diagnosis; PGS, preimplantation genetic aneuploidy screening.

has also been observed between the number of failed IVF attempts and the numerical chromosomal abnormalities (50% with three and 67% with more than five) (Gianaroli *et al*., 1997).

Although data on recurrent implantation failure are limited, most studies have failed to demonstrate improved clinical outcomes after PGS (18% pregnancy rate per oocyte retrieval) (Harper *et al*., 2006), particularly in older women (>38 years) with more than two previously failed cycles (Munné *et al*., 2003). However, some studies (Kahraman *et al*., 2000; Pehlivan *et al*., 2003) have observed better results in young women (mean age 30 ± 3.1 years: 30.4% ongoing pregnancy rate; mean age 33.7 ± 1.6: 40.7% pregnancy rate, respectively). The most important flaws of these two studies are that both used an inappropriate control group. Kahraman *et al*. compared the outcome of recurrent implantation failure women with advanced maternal age women and Pehlivan *et al*. included couples undergoing PGD for sex-linked diseases without recurrent implantation failure (Kahraman *et al*., 2000; Pehlivan *et al*., 2003). In addition, Platteau *et al*. found a live birth rate of 29.7% in a descriptive study including 121 women of <37 years (Platteau *et al*., 2006). On the contrary, in a series of 54 young patients (mean 32 ± 2.3 years) who decided to undergo either assisted hatching or PGS, no benefit on their implantation and clinical pregnancy rates was found (17.3 and 25%, respectively) (Gianaroli *et al*., 1999).

The only randomized trial conducted so far evaluated only 19 patients with recurrent implantation failure and concluded that PGD offered no benefit to these couples (Werlin *et al*., 2003).

NOA and OA

An increased aneuploidy rate has been observed on both testicular and epididymal spermatozoa from NOA and OA compared with normozoospermic men (19.6 versus 8.2 versus 1.6%, respectively) (Calogero *et al*., 2003). This has been attributed to meiotic spindle disorders because of disturbed intra-testicular environment or the existence of gene mutations (Egozcue *et al*., 2000). Increased aneuploidy and mosaicism rates have also been demonstrated on embryos derived from azoospermic men (NOA and OA) compared with embryos derived from fertile men (Silber *et al*., 2003; Platteau *et al*., 2004; Rubio *et al*., 2005), which constitutes the basis for proposing the performance of aneuploidy screening on these men.

Unfortunately, hardly any data are available in this group of patients. Rubio *et al*. retrospectively evaluated the results of PGS in 20 cycles in OA and 18 cycles in NOA and compared them with a group of PGD for sex-linked disorders. No statistically significant difference was observed in either implantation or pregnancy rates (Rubio *et al*., 2005).

Severe sperm morphology anomalies

Multiple studies have documented an enlarged frequency of sperm chromosomal anomalies in teratozoospermic spermatozoa (Bernardini *et al*., 1998; Calogero *et al*., 2003; Vicari *et al*., 2003), thereby generating controversy on whether these patients should undergo ICSI. Although infrequent, macrocephalic spermatozoa have been more extensively studied in the context of PGS, because they seem to be more strongly related to aneuploidy (Viville *et al*., 2000; Devillard *et al*., 2002). Moreover, lower fertilization and pregnancy rates have been reported after ICSI, even when compared with patients with a complete absence of normal sperm morphology (43 and 9%, respectively) (Kahraman *et al*., 1999). Kahraman *et al*. retrospectively compared the outcome after PGS on both predominantly macrocephalic and other sperm morphological anomalies

and found a higher implantation rate only in the macrocephalic group (25 versus 12.3%) (Kahraman *et al*., 2004).

What are the limitations of PGS?

Although the performance of aneuploidy screening to improve IVF outcome is based on solid theoretical grounds, several disadvantages constitute major limitations for its clinical value.

Mosaicism

Mosaicism is defined either by the presence of euploid and aneuploid cells or distinct aneuploidies on different blastomeres and it has been found in up to 57% of day 3 biopsied embryos (Baart *et al*., 2004b; Coonen *et al*., 2004). These embryos are the consequence of mitotic errors in a diploid zygote secondary to non-disjunction or anaphase lagging. It has been reported that anaphase lagging is responsible for 56% of the mosaicism observed in the blastocyst stage (Coonen *et al*., 2004). Although mitotic errors have not been regarded as age related, Munné *et al*. have described a maternal age increase on mosaicism derived from mitotic non-disjunction, which could be attributed to a malfunction of the cellular apparatus (Munné *et al*., 2002).

Different types of mosaic embryos have been described. The combination of diploid and chaotic cells is the most frequently encountered in early stage embryos, followed by diploid and trisomic or monosomic cells (Bielanska *et al*., 2002). Regarding blastocysts, some authors have observed that complex mosaicism is the most prevalent type (31%) (Coonen *et al*., 2004), whereas others have pointed to the diploid–polyploid combination (67%) (Bielanska *et al*., 2002). Table IV summarizes the types and frequencies of mosaicism observed in cleavage stage embryos when at least two blastomeres are biopsied.

Mosaicism may represent a major source of misdiagnosis (60%) in PGS (Munné *et al*., 2002) because of both false-positive and false-negative results. This is especially the case when only one cell is analysed, as an anomaly of meiotic origin cannot be distinguished from a mosaic embryo. When re-analysis of good quality day 3 biopsied embryos is performed on day 5, low cytogenetical confirmation rates have been encountered, even after the assessment of two blastomeres (35%) (Baart *et al*., 2004b). The clinical relevance of the former is the loss of euploid embryos that will be discarded for transfer.

Even though the fate of mosaic embryos is as yet not well understood, their developmental potential seems to be related to the proportion and type of aneuploid cells involved. It has been reported that when polyploid mosaic embryos have less than 38% of abnormal cells, there is a significant increase in the number of embryos developing to the blastocyst stage (78 versus 33%, respectively) (Sandalinas *et al*., 2001). Regarding the types of mosaic embryos, chaotic mosaicism, defined as the existence of multiple chromosome anomalies on different cells, has shown the highest rate of developmental arrest. On the contrary, polyploid mosaicism reveals the lowest chance of arrest (Bielanska *et al*., 2002). Nevertheless, based on the low incidence (5%) of mosaicism encountered in spontaneous abortions and vital pregnancies (2%), it is likely that most mosaic embryos are eliminated before the first trimester of pregnancy (Los *et al*., 2004). This is probably initiated after the activation of the embryonic genome (8-cell stage), resulting in both the arrest of already developed mosaic embryos or the prevention of its further development by discarding the abnormal cells.

The complexity of the process arises from the presence of dynamic changes through *in vitro* development, either by the correction of existent anomalies or by the emergence of new ones. Even the normalization of trisomic embryos after re-analysis on day 12 has been recently described (Munné *et al*., 2005). Three mechanisms of correction were proposed: anaphase lagging, non-disjunction and chromosome demolition. It can be questioned, however, whether these embryos are genetically normal because of the existence of uniparental disomy (Robinson, 2000).

Although biological variables are extremely relevant, technical issues must also be considered. Because the biopsy is not randomly performed, when two blastomeres are removed there is ~25% chance of extracting both reciprocal daughter cells, thereby transforming a mosaic embryo into a euploid status (Baart *et al*., 2006). It has also been proposed that in some cases of low-grade mosaicism, abnormal cells could migrate towards the trophectoderm, although this has recently been refuted as a similar proportion of abnormal cells has been found on the inner cell mass (Derhaag *et al*., 2003). These studies have all been conducted after ovarian stimulation and IVF, and these conditions may not represent physiology (Munné *et al*., 1997). Moreover, most of the information derives from embryos that have been either discarded for transfer or cryopreserved, nevertheless, current available data are consistent in demonstrating high rates of mosaicism in cleavage stage embryos. Therefore, until proven wrong, this

Table IV. Mosaicism rates in different group of patients when two blastomeres are biopsied

Study	Patients	Mosaicism rate (%)			Type of mosaicism (%)
Bielanska *et al*. (2002)	Embryos donated for research by 81 patients (n = 216 embryos)	48.1			Diploid–chaotic: 16.2; diploid–aneuploid: 14.4; diploid–haploid: 2.8; diploid–polyploid 14.8
Pehlivan *et al*. (2003)	Recurrent implantation failure (≥3)	<37	>37	Control	Not stated
	<37 years (n = 27)	20.5	18.9	10.8	
	≥37 years (n = 22)				
	Control PGD sex-linked disease <37 years (n = 9)				
Staessen *et al*. (2004)	Age ≥37 years (n = 653 embryos)	10.4			Diploid–monosomy: 5; diploid–trisomy: 3.1; diploid–combined abnormalities: 1.1; monosomy–trisomy 1.2
Baart *et al*. (2006)	Age <38 years (n = 60)	50			Diploid–abnormal: 28; abnormal–abnormal: 22

valuable information should be considered when interpreting PGS results.

Misdiagnosis

Besides mosaicism, several technical limitations have been described. Overlapping signals may be a source of misdiagnosis resulting in false diagnosis of monosomies, which is associated with the number of used probes and the type of labelling technique (4–8%). This source of misdiagnosis can be reduced using different fluorochromes for each chromosome instead of ratio labelling (Bahce *et al*., 2000). Signal splitting has also been described, resulting in the detection of false trisomies. It has been observed that probes for chromosomes X, 13, 16 and 21 produce fewer errors than those for Y and 18 (Munné *et al*., 2002).

The number of analysed cells also remains an important issue subject to debate. The removal of two blastomeres has been shown to render a higher proportion of analysable embryos compared with the removal of only one blastomere (98.2 versus 95.9%.); however, this difference is probably not clinically significant (Michiels *et al*., 2006). After re-analysis of non-transferred embryos, a higher correlation rate for aneuploidy was found when two blastomeres were compared with one (82 versus 58%) (Baart *et al*., 2006). In a recent study that compared the diagnostic accuracy of single versus double blastomere biopsy in 1888 embryos, no significant difference was observed in sensitivity (100 versus 100%) and specificity (74.4 versus 86.4%) but the positive likelihood ratio was higher when two single spread nuclei were available (7.35 versus 3.9) (Michiels *et al*., 2006). A trend towards a higher proportion of embryos falsely diagnosed as abnormal was also reported after the analysis of only one nuclei compared with two (8.1 versus 3.3%) (Michiels *et al*., 2006), resulting in the loss of embryos with a chance of implantation. One possible disadvantage of removing two blastomeres is a higher risk of aggravation of mosaicism, which may convert a potentially successful embryo without testing into a non-viable but transferable embryo after testing (Los *et al*., 2004).

Nonetheless, misdiagnosis remains infrequent. The ESHRE PGD Consortium data collection has so far reported only three cases of misdiagnosis by using FISH (Sermon *et al*., 2005). In addition, six more misdiagnoses of trisomy 21 have been documented separately (Gianaroli *et al*., 2001; Verlinsky *et al*., 2004; Munné *et al*., 2006).

Does PGS provide prognosis information?

It has recently been suggested that PGS could also be used as a prognostic tool in assisting in the counselling of patients with advanced maternal age and recurrent IVF failure (Ferraretti *et al*., 2004). When no euploid embryo was available in the first cycle of treatment, the chance of finding a chromosomally normal embryo in a subsequent cycle was significantly reduced compared with patients for whom one or two euploid embryos were available (8.4 versus 22.3 versus 32.4%). These patients also had a significantly lower live birth rate (8.5 versus 30%). According to these data, PGS can provide important information for these couples, either by encouraging them to continue the treatment if there is at least one euploid embryo available for transfer or by advising them to undergo oocyte donation when only aneuploid embryos are encountered.

Possible future trends in PGS

Comparative genomic hybridization

CGH performed on a single cell basis constitutes a recently developed technology that enables the assessment of all the chromosomes by comparing the studied DNA with a normal sample. Furthermore, CGH has been able to identify chromosome breakages non-detectable by using FISH (Voullaire *et al*., 2000).

In brief, both DNA samples are labelled with red (normal DNA) and green fluorochromes (test DNA) and then applied to a slide covered with normal human metaphase chromosomes, where hybridization occurs for 48–72 h. For single cell analysis purposes, several amplification methods have been developed (Wilton, 2005). So far, the rates of normal embryos have been shown to be lower than when FISH analysis is performed (~25%) (Voullaire *et al*., 2000; Wells and Delhanty, 2000; Wilton *et al*., 2003).

One limitation of CGH is that it cannot distinguish diploid cells from haploid or tetraploid (Wilton, 2005). The long period required for hybridization (5 days) has limited the widespread clinical implementation of this technique, as it is necessary to freeze all the embryos after the biopsy. This approach would lead to an important decrease in success rates as it has been demonstrated that there is a significant reduction in the survival rate of biopsied embryos after cryopreservation (Joris *et al*., 1999; Magli *et al*., 1999). Despite this, using a modified freezing protocol, the birth of the first child after CHG performance has already been documented (Wilton *et al*., 2001). The development of new techniques with the implementation of microarrays might also help to surpass this limitation by shortening the time required for hybridization (Hu *et al*., 2004).

Blastocyst biopsy

The performance of chromosomal assessment in the blastocyst stage offers two significant advantages: an enhanced detection of mosaicism by evaluating more cells and an additional embryo selection derived from *in vitro* development (De Boer *et al*., 2004).

Embryos are hatched on day 3 to facilitate the herniation of trophectoderm cells and, on day 5, five to six cells are extracted for analysis. The largest published series to date has reported 173 biopsied embryos in 63 cycles performed for aneuploidy screening in patients with recurrent implantation failure, corresponding to an implantation rate per embryo transferred of 30% (McArthur *et al*., 2005). Nevertheless, there is still need for more data to confirm these encouraging results. The most significant unanswered questions concern the safety of this technique and whether trophectoderm cells truly represent the inner cell mass and future fetus.

Conclusions

PGS has been performed during the last decade to improve embryo selection in patients with a poor reproductive outcome associated with a high frequency of embryonic numerical chromosome abnormalities. Nevertheless, major technical limitations as well as a lack of consistent evidence in the literature have not yet enabled definitive conclusions to be drawn. Therefore, scientific efforts must be performed to increase our knowledge and so also improve our patient counselling. The design of further studies

should include an adequate randomization protocol with a clear stratification concerning the indications to perform PGS, the replacement of the same number of embryos in both study and control groups and the healthy live birth rate per treatment cycle as the main outcome measure. Expanding our knowledge concerning the incidence of embryo numerical chromosomal abnormalities in young women undergoing IVF as well as in natural cycles can also provide a better understanding of the influence of age and ovarian stimulation on the emergence of aneuploid embryos.

Mosaicism constitutes the most significant drawback for the clinical application of PGS, as it can lead to misdiagnosis, thereby reducing its efficiency. The development of new technologies that enable a complete assessment of the numerical chromosomal constitution of preimplantational embryos as well as an enhanced recognition of mosaicism might help overcome some of the current limitations of FISH and offer new insights into the value of PGS.

The current follow-up of children born after PGS indicates, so far, no detrimental effect of the biopsy, as no differences have been reported when compared with conventional ICSI cycles. The data are, however, limited as only 648 pregnancies have been followed after PGD (Harper *et al*., 2006). Verlinsky *et al*. have also reported on the follow-up of 754 babies born after 4748 PGD cycles without a significant increase on the prevalence of congenital malformations (Verlinsky *et al*., 2004).

According to observational studies, performing PGS in advanced maternal age women yields higher implantation rates and a reduced risk of miscarriage. When considering the evidence provided by randomized trials, however, PGS does not demonstrate an outcome improvement when there is no limitation in the number of embryos transferred. In patients with recurrent implantation failure and recurrent miscarriage, there is insufficient evidence to support a beneficial effect of PGS. In addition, considering the risks and costs of undergoing IVF and PGD, this technique should not be implemented on a routine basis. In the case of azoospermic men and severe sperm morphology anomalies, more research is needed to determine if there is a beneficial effect. Additional evidence is therefore needed before aneuploidy screening can be implemented in routine clinical practice.

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