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Preimplantation genetic diagnosis: design or too much design

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Abstract

Preimplantation genetic diagnosis (PGD) is a technique that was first applied in humans in 1990 (Handyside *et al*., 1990; Verlinsky *et al.*, 1990). Thirty years on an estimated 15000 children have been conceived and born using PGD, a number dwarfed by the huge number of children already conceived via conventional in vitro fertilisation. In contrast to numerous reports on reproductive outcome in conventional IVF, data on reproductive outcome of PGD are scarse. There is ongoing debate about the diagnostic accuracy and clinical relevance of Preimplantation genetic screening for aneuploidy (PGS) (Shahine *et al.,* 2006; Twisk *et al.,* 2006), however well conducted prospective randomized studies are few. In this PhD summary, the author describes the reproductive results of a large PGD program and applies life table analysis with multiple regression analysis and comparative analysis where appropriate. Potential risks of PGD including misdiagnosis, perinatal mortality and monozygotic twinning rate are assessed. The aim is to provide both patients and physicians with adequate information on all reproductive aspects of PGD as a diagnostic and therapeutic tool.

Key words: PGD, PGS, ICSI, delivery rate, preimplantation genetic diagnosis.

Introduction

Preimplantation genetic diagnosis (PGD) is a procedure used by fertile or infertile couples at high risk of transmitting a genetic condition and allows diagnosis of single gene disorders, chromosomal abnormalities or HLA typing in embryos prior to transfer and implantation. In this way it offers an alternative to prenatal diagnosis by chorion villus sampling (CVS) or amniocentesis (AC) and termination of pregnancy. Preimplantation genetic screening for aneuploidy (PGS) is a related procedure that is offered to infertile couples with advanced age of the female partner, previously failed in vitro fertilization (IVF) treatment or unexplained recurrent miscarriage, with the aim of improving the success rate of IVF. It allows the enumeration of chosen chromosome pairs and can be considered as an early form of prenatal aneuploidy screening. Ever since the first application of the technique in 1990 (Handyside *et al.*, 1990; Verlinsky *et al.*, 1990), the number of indications for PGD has increased considerably, as has the number of couples filing a request, and over 15000 cycles of PGD/PGS have been registered in Europe alone, resulting in over 2000 births (Sermon *et al.*, 2004; Goossens *et al.*, 2008).

The patients that attend the PGD clinic are often not aware of the risks and benefits, the pros and cons, the larger implications of a treatment with ovarian stimulation, oocyte retrieval, intracytoplasmic sperm retrieval (ICSI) and PGD. Many patients requesting PGD do not suffer from infertility. They request PGD for the mere goal of avoiding their children to be affected or carrier of a specific genetic disorder, or to eradicate a genetic condition from their family. Patients requesting PGS on the other hand, are very much aware of their infertile status, and aim to increase pregnancy rate and avoid miscarriages and trisomic children by genetic selection of their embryos. However they are equally not always informed about the risks and limitations of such techniques. The analyses and results presented in this overview are aimed at providing patients requesting PGD or PGS correct information on reproductive

outcome, contribution to that outcome by identifiable factors and risks associated with this technique.

Indications for PGD

The most common indication for PGD is cystic fibrosis (CF) (Gutiérrez-Mateo *et al.*, 2009), which was the first monogenic disorder to be diagnosed by PGD (Handyside *et al.*, 1992) A list of most common indications is shown in Table 1. Most couples requesting PGD for CF do so because they have been identified as carriers at screening prior to reproductive treatment. The close interface between genetics and reproductive medicine is illustrated by the fact that a number of these couples have been unsuccessful at conceiving due to obstructive azoospermia secondary to congenital bilateral absence of the vas deferens (CBAVD) which is shown to be associated with carrier or affected status of CF in at least 80% of cases (Lissens *et al.*, 1996). Another challenge for reproductive physicians and geneticists is the reproductive treatment with PGD of CF affected women who often present with significant health problems and use of a significant amount of medication.

In the population studied in current PGD practice at our centre, the most common indications for PGD are myotonic dystrophy type 1 (syn: dystrophia myotonica type 1; DM1; Steinert's disease; OMIM #160900), Huntington disease (OMIM +143100) and Fragile X syndrome (OMIM #300624) (for a list of all indications see Table 2). This selection is mainly due to the expertise developed in the detection of triplet repeat disorders at single cell level, rather than a high incidence of these disorders in the Flemish population. Approximately 30% of the population requesting PGD comes from abroad. The relevance of studying reproductive outcome in this population is again illustrated by the fact that triplet repeat disorders are commonly associated with infertility problems, including poor sperm quality in DM1 men and risk of premature ovarian failure in female fragile X carriers, more in particular those with premutations in the Fragile X mental retardation protein (FMRP) gene (Platteau *et al.*, 2002).

PGS is a technique allowing chromosomal aneuploidy analysis by fluorescence in situ hybridization (FISH) in pre-transfer embryos following in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI), and can be considered as an early form of prenatal screening for numerical chromosomal abnormalities. Many studies have argued a potential benefit of PGS in couples at high risk of chromosomally abnormal embryos, including in cases of advanced maternal age (Gianaroli *et al.*, 1999; Kuliev *et al.*, 2003; Munné *et al.*, 2003; Platteau *et al.*, 2005a), recurrent miscarriage (Pellicer *et al.*, 1999; Rubio *et al.*, 2005; Gianaroli *et al.*, 2005; Munné *et al.*, 2005; Platteau *et al.*, 2005b) and recurrent implantation failure (Pehlivan *et al.*, 2003; Wilding *et al.*, 2004), whereas other authors have not

Table 1. — Most common indications for PGD or PGS.

preimplantation genetic diagnosis (PGD)	
1. Autosomal recessive	cystic fibrosis
	beta-thalassaemia
	spinal muscular atrophy
	sickle-cell anaemia
2. Autosomal dominant	epidermolysis bullosa
	myotonic dystrophy type 1
	Huntington's disease
	amyloid polyneuropathy
	Charcot-Marie-Tooth disease
	achondroplasia
	Marfan's syndrome
3. Specific sex-linked	Duchenne muscular dystrophy
	fragile-X syndrome
	haemophilia
4. Structural chromosomal abnormalities	robertsonian translocations
	reciprocal translocations
	sex chromosome aneuploidy
preimplantation genetic screening (PGS)	
1. Advanced maternal age	
2. Recurrent implantation failure	
3. Recurrent miscarriage	
4. Severe male factor infertility	

Table 2. — Indications for PGD in the study cohort 1993-2005 at UZ Brussel PGD clinic.

indication	OMIM reference number(s) disorder	OMIM nr. + name of gene / genetic region tested	method	N patients
Autosomal Dominant				
achondroplasia	#100800	*134934 (FGFR3)	PCR	2
autosomal dominant polycystic kidney disease (ADPKD)	#173900	+601313 (PKD1)	PCR	3
breast cancer 1 gene (BRCA1)	#114480	*113705 (BRCA1)	PCR	2
Charcot Marie Tooth (CMT) type 1A	#118220	*601097 (PMP22)	PCR	9
Dystonia Musculorum Deformans 1	#128100	*605204 (DYT1)	PCR	1
Ectrodactyly Ectodermal Dysplasia and orofacial clefts (EEC3)	#604292	*603273 (TP63)	PCR	1
Epidermolysis Bullosa Simplex DOWLING-MEARA TYPE	#131760	*148066 (KRT14)	PCR	1
Familial Adenomatous Polyposis of the Colon (FAP)	#175100	*611731 (APC)	PCR	2
Facioscapulohumeral dystrophy (FSHD)	158900%	No Omim Reference (4q35)	PCR	4
Huntington Disease exclusion	+143100	+143100 (HD)	PCR	16
Huntington Disease (HD)	+143100	+143100 (HD)	PCR	37
Hypokalemic periodic paralysis (HOKPP)	#170400	*114208 (CACNA1S)	PCR	2
Marfan syndrome	#154700	*134797 (FBN1)	PCR	8
Multiple Endocrine Neoplasia type 2A (MEN2A)	#171400	+164761 (RET)	PCR	1
Multiple Exostoses (EXT1)	#133700	*608177 (EXT1)	PCR	1
Myotonic Dystrophy 1 (DM1)	#160900	*605377 (DMPK)	PCR	78
Neurofibromatosis type 1 (NF1)	+162200	+162200 (NF1)	PCR	8
Neurofibromatosis type 2 (NF2)	#101000	*607379 (NF2)	PCR	1
Osteogenesis Imperfecta (OI) type I	#166200	+120150 (COL1A1)	PCR	3
Osteogenesis Imperfecta (OI) type IV	#166220	*120160 (COL1A2)	PCR	2
Retinoblastoma	+180200	+180200 (RB1)	PCR	1
Spinocerebellar Ataxia 1 (SCA1)	#164400	*601556 (ATXN1)	PCR	1
Spinocerebellar Ataxia 7 (SCA7)	#164500	*607640 (ATXN7)	PCR	3
Stickler syndrome type I	#108300	+120140 (COL2A1)	PCR	1
Tuberous Sclerosis type 1	#191100	*605284 (TSC1)	PCR	4
Tuberous Sclerosis type 2	#191100	*191092 (TSC2)	PCR	1
Autosomal Recessive				
ARPKD	#263200	*606702 (PKHD1)	PCR	2
21 hydroxylase deficiency	+201910	+201910 (CYP21)	PCR	1
Beta-thalassemia	+141900	+141900 (HBB)	PCR	5
Canavan disease	#271900	*608034 (ASPA)	PCR	1
Carbohydrate Deficient Glycoprotein syndrome (CDG) type Ia	#212065	*601785 (PMM2)	PCR	1
Carbohydrate Deficient Glycoprotein syndrome (CDG) type Ic	#603147	*604566 (ALG6)	PCR	1
Cystic fibrosis (CF)	#219700	*602421 (CFTR)	PCR	64
Connexin 26 deafness	#220290	*121011 (GJB2)	PCR	2
Familial Dysautonomia (DYS)	#223900	*603722 (IKBKAP)	PCR	1
Gaucher disease type II	#230900	*606463 (GBA)	PCR	2
Glutaric Acidemia type I	#231670	*608801 (GCDH)	PCR	1
Glycogenosis	+232200	+232200 (G6PC)	PCR	1
Medium Chain AcetylCoA dehydrogenase (MCAD) deficiency	#201450	*607008 (ACADM)	PCR	1
Pompe disease	#232300	*606800 (GAA)	PCR	1
Rhizomelic Chondrodysplasia Punctata type 1	#215100	+601757 (PEX7)	PCR	1
Sickle Cell Anaemia	#603903	+141900 (HBB)	PCR	6
Spinal Muscular Atrophy (SMA) types I, II, III	#253300, #253550, #253400	*600354 (SMN1)	PCR	17
Non-Mendelian				
Leber hereditary optic neuropathy (LHON)	#535000		FISH-sexing	1
Renal Agenesis	undefined		FISH-sexing	1

Table 2. — Continuation.

X-linked recessive				
Adrenoleukodystrophy	#300100	X-chromosome (sexing) or *300371 (ABCD1)	FISH or PCR	4
Adrenoleukomyeloneuropathy	#300100	X-chromosome (sexing)	FISH	1
Agammaglobulinemia	#300755	*300300 (BTK)	PCR	1
Alport syndrome	#301050	X-chromosome (sexing)	FISH	4
Androgen insensitivity syndrome	#300068	*313700 (AR)	PCR	1
Choroideremia	#303100	*300390 (CHM)	PCR	1
Duchenne Muscular Dystrophy	#310200	X-chromosome (sexing) or *300377 (DMD)	FISH or PCR	25
Ectodermal dysplasia, Hypohydrotic, x-linked	#305100, #300291	X-chromosome (sexing)	FISH	2
Fabry Disease	#301500	X-chromosome (sexing)	FISH	1
FG syndrome	not defined for this patient	X-chromosome (sexing)	FISH	1
Hemophilia A	+306700	X-chromosome (sexing)	FISH	11
Hydrocephaly	#307000	X-chromosome (sexing)	FISH	3
Kallmann syndrome	+308700	X-chromosome (sexing)	FISH	1
Kennedy disease	#313200	*313700 (AR)	PCR	2
Menkes disease	#309400	X-chromosome (sexing)	FISH	2
Myotubular Myopathy	#310400	X-chromosome (sexing)	FISH	1
Ornithine Transcarbamylase Deficiency (OTC)	#311250	X-chromosome (sexing)	FISH	1
Retinitis Pigmentosa type III	#300389	X-chromosome (sexing)	FISH	8
Retinoschisis 1	+312700	X-chromosome (sexing)	FISH	1
Severe Combined Immunodeficiency (SCID)	#300400	*308380 (IL2RG)	PCR	1
Wiskott-Aldrich syndrome (WAS)	#301000	X-chromosome (sexing)	FISH	2
X-linked mental retardation (MR)	Heterogeneous group	X-chromosome (sexing)	FISH	7
Charcot Marie Tooth X linked	not defined for this patient	X-chromosome (sexing)	FISH	1
Chondrodysplasia punctata	not defined for this patient	X-chromosome (sexing)	FISH	1
X-linked dominant				
Incontinentia Pigmenti (IP)	#308300	*300248 (IKBKG; = NEMO)	PCR	3
Oro facial Digital Syndrome I (OFD 1)	#311200	X-chromosome (sexing)	FISH	2
Fragile X syndrome	#300624	*309550 (FMR1)	PCR	37
Fragile X syndrome + Mental retardation	#300624 + X-linked MR	*309550 (FMR1) + Unknown	FISH + PCR	1
HLA typing				
Beta-thalassemia + HLA	+141900	+141900 (HBB), HLA	PCR	3
Chronic septic granulomatosis + HLA	#306400	X-chromosome (sexing), HLA	FISH + PCR	1
Fanconi anaemia + HLA	#227650	*607139 (FANCA), HLA	PCR	2
Leukemia + HLA	Not relevant	HLA	PCR	5
Sickle Cell Anaemia + HLA	#603903	+141900 (HBB), HLA	PCR	5
Wiskott-Aldrich syndrome (WAS) + HLA	#301000	*300392 (WAS), HLA	PCR	1
Robertsonian translocations				57
reciprocal translocations				90
other chromosomal abnormalities				68
PGS				837

OMIM = Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/omim).

been able to find an unequivocal benefit (Staessen *et al.*, 2004; Mastenbroek *et al.*, 2007) or have found restrictions to the clinical benefit when a low number of embryos is available for analysis (Munné *et al.*, 2003; Platteau *et al.*, 2005a). The clinical benefit of PGS in improving live birth rate may therefore be under scrutiny, but this technique may appear to be useful in improving selection of euploid embryos, thereby reducing implantation failure and miscarriage rates (*for review see* Donoso P *et al.*, 2007).

Reproductive techniques used in PGD

In our program, pituitary desensitisation was carried out in an agonist protocol, using GnRH analogues (buserelin, Suprefact°; Hoechst, Frankfurt, Germany), in combination with ovarian stimulation with human menopausal gonadotrophins (hMG) (Menopur°, Ferring Pharmaceuticals A/S, Copenhagen, Denmark) or recombinant FSH (Puregon°, Schering-Plough, Oss, The Netherlands) (Van de Velde *et al.*, 1998), or in an antagonist protocol with a GnRH antagonist (ganirelix, Orgalutran°, Schering-Plough) combined with recombinant FSH or hMG (Kolibianakis *et al.*, 2004). The starting dose of gonadotrophins was based on the female partner's age, preliminary ovarian response assessment and/or previous response to ovarian stimulation (range 75–450 IU). Human chorionic gonadotrophin (hCG) (10000IU, Pregnyl; Schering-Plough or Profasi°, Merck-Serono, Geneva, Switzerland) was administered for final oocyte maturation. Transvaginal ultrasound-guided oocyte collection (OC) was scheduled 36 hours after hCG administration. Oocyte collection (OC) was carried out under premedication with pethidine 1 mg/kg IM and paracervical block with mepivacaine hydrochloride, or under general anaesthesia as and when indicated (Van de Velde *et al.*, 1998, Kolibianakis *et al.*, 2004).

The details of the IVF and ICSI procedure have been described previously (Van Landuyt *et al.*, 2005). In the event of PGD/PGS, ICSI was the method of choice rather than classical IVF to prevent contamination with residual sperm DNA in case of PCR-based PGD (Liebaers *et al.*, 1998) and to maximise the fertilisation rate in PGS. Fertilisation was assessed 16 to 18 hours after ICSI. Further development was evaluated in the morning of day two and again on day three, when embryos are evaluated before biopsy. According to the number of anucleate fragments, the embryos are subdivided into grades A, B, C and D as described previously (Vandervorst *et al.*, 1998). From the 5-cell stage onwards for FISH analysis and from the 6- cell stage onwards, embryo biopsy of grade A, B and C performed on day 3 of culture. In the initial stages of PGD at our centre, an acid solution (Tyrode's solution) was used to breach the zona pellucida. Laser assisted biopsy has been applied since June 1999 as previously described (De Vos *et al.*, 2001; Sermon, 2002). Overall the aspiration method was used to remove one or two blastomeres from the embryo (Fig. 1, 2). For PCR analysis, each blastomere was placed in a solution that lyses the cell and releases the DNA (Sermon *et al.*, 2004). For Fluorescence In Situ Hybridisation (FISH) purposes, a blastomere was spread on a slide using the HCl/Tween 20 method (Coonen *et al.*, 1994).

Genetic techniques used in PGD

The polymerase chain reaction (PCR) procedures were performed as previously described (Sermon *et al.*, 2001; Sermon and De Rycke, 2007). Multiplex PCR is the simultaneous amplification of two or more DNA sequences, and over the years it has become the standard method of DNA amplification at single cell level, reducing both the risk of undetected contamination and allele drop out (ADO) by the use of linked markers alone, or linked markers combined with the detection of a specific mutation (Sermon, 2002).

Numerical chromosomal analysis was performed using a FISH procedure, allowing analysis of chromosomes X and Y, and depending on the availability of the fluorochromes at the time of analysis, for chromosome 18, for chromosomes 13 and 21, and at even later stages also for chromosomes 16 and 22 in a second round of hybridisation (Staessen *et al.*, 1999, 2004). For reciprocal translocations, the direct labelled and commercially available probes (from Vysis or Cytocell) consisted of a combination of three probes, one telomeric and two centromeric, or two telomeric and one centromeric of the chromosomes involved. For Robertsonian translocations, we use a combination of either two subtelomeric or two locus-specific probes or a combination of a subtelomeric and a locus-specific probe. By this approach, the embryos carrying normal or balanced chromosomes can be differentiated from the embryos carrying unbalanced chromosomes.

Statistical tools used to assess reproductive outcome in PGD

The statistical methods used in the data analysis leading to this thesis, have shown both strengths and weaknesses.

The aim of the thesis was in the first place to be descriptive on the reproductive outcome in couples undergoing IVF or ICSI associated with PGD. The cumulative live birth delivery rate using Kaplan-Meier analysis expresses the calculated chance of

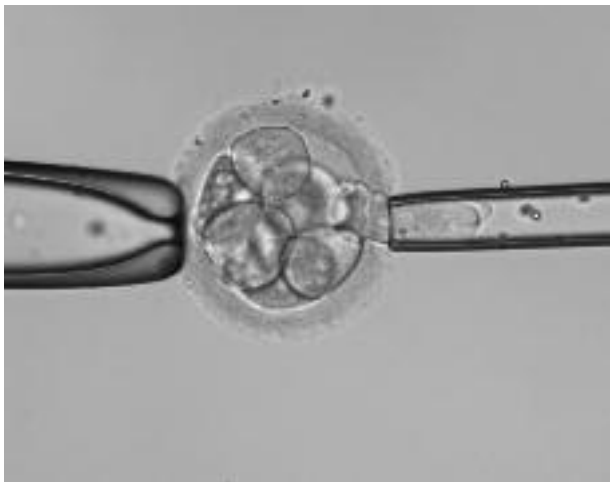

Fig. 1

Fig. 2

having at least one child following IVF/ICSI + PGD/PGS, on the basis that those couples who started the treatment, completed 6 treatment cycles, and that those couples who stopped the treatment during the course of this period, were included in the cumulative analysis assuming a reproductive prognosis similar to those who in actual fact continued the treatment.

Most reports and registries use pregnancy and birth rates per started cycle, oocyte retrieval or embryo transfer as primary outcome parameter (or miscarriage rate per cycle) as primary adverse outcome parameter. This is in line with the definition of clinical pregnancy rate and especially live birth delivery rate as set out by the ICMART glossary (Zegers-Hochschild *et al.*, 2006). With increasing evidence of the effectiveness of ART, reproductive outcome has been reported on a per-cycle basis rather than a per-patient basis (Daya, 2005). The outcome of a single cycle is of interest, but only as part of the whole treatment in the overall context of patient discomfort, complications and costs (Heijnen *et al.*, 2004). In the current climate of legal restriction on number of embryos transferred under IVF reimbursement laws, as is the case in Belgium (Ombelet *et al.*, 2005; De Neubourg *et al.*, 2006), and a trend towards milder stimulation to avoid complications such as ovarian hyperstimulation, a term singleton delivery is the ideal outcome. Fortunately, and as a result of multiple publications on reproductive outcome analysis, outcome reporting has shifted from biochemical, clinical and ongoing pregnancy rates to live birth rate, delivery rate or by preference singleton term live birth rate per cycle (Fauser *et al.*, 2002; Daya, 2003; Heijnen *et al.*, 2004; Min *et al.*, 2004) or even singleton live birth rate per oocyte as the benchmark prognostic parameter (Pinborg *et al.*, 2004). Because ART treatments are relatively short in duration and several cycles are often needed to succeed, the cumulative delivery rate using life table analysis is frequently employed to estimate the effectiveness of treatment and guide clinical decision-making (Daya, 2005).

The life table method was originally devised for analysing light bulb failure times and was then applied clinically for analysing death rates, so that failure rates resulting from cancer treatment could be ascertained and survival times could be estimated (Berkson and Gage, 1950). This method, usually known as survival analysis, was a significant improvement over the approach of using gross death rates, because it incorporated the actual rates of death as observed at various time points following diagnosis or commencement of treatment. Moreover, this method does not require subjects to enter simultaneously into a study or database, and can make use of data of subjects who dropped out of the study or who were lost for follow-up. The most important aspect of the life table method is the incorporation in the survival analysis of the duration of the time taken to reach the outcome event.

Cumulative outcome analysis (aka life table analysis; survival analysis) is rarely performed in reproductive medicine, for several reasons. The most important reason is that cumulative delivery rate calculation is only possible if drop out cases are non-informative, i.e. if there is no identified reason why patients discontinued the treatment after (n) cycles. Informative censoring may introduce bias into the standard methods used for survival analysis. In reproductive medicine it is assumed that patients discontinue the treatment cycles on the basis that the outcome is limited on medical grounds, be it poor gamete quality, failed fertilisation, poor embryo

development, or associated reasons. Although this will surely feature in the group of drop out patients in the PGD population, we assume that a large number of PGD patients discontinue treatment on non-identified and therefore non-informative grounds such as financial reasons, PGD treatment being expensive, ill health not associated with the reproductive status, poor access to the PGD services provided at our centre for patients coming from abroad, and other non-informative reasons.

A second reason why cumulative outcome analysis is rarely performed in reproductive medicine is the fact that the period over which reproductive outcome is assessed is not time, but a number of treatment cycles performed, classically six cycles. The variability in the number of ART cycles couples may undertake and the length of time they may have to wait between successive cycles of treatment contribute to the complexity of assessing effectiveness of ART (Daya, 2005).

A third reason is that patient groups per centre are usually small, in comparison to the large patient groups in oncology where survival analysis was applied.

As Witsenburg et al mentioned in their manuscript on cumulative live birth rates in IVF and ICSI, others have already reported cumulative (live) birth rates or pregnancy rates after multiple IVF/ICSI treatments (Alsalili *et al.*, 1995; Dor *et al.*, 1996; Engmann *et al.*, 1999), whereby the authors usually describe both (expected) cumulative delivery rate and observed (crude, true) cumulative delivery rate, while they prefer the expected rate (Osmanogaoglu *et al.*, 1999; Fukuda *et al.*, 2001; Witsenburg *et al.*, 2005).

Alternatives to reporting reproductive outcome in ART by survival analysis are 1. pregnancy rate per cycle, 2. time-limited analysis using proportions, 3. conservative cycle-based cumulative pregnancy rate, and 4. real-time based cumulative pregnancy rate, of which the latter is recommended as the best option (Daya, 2005).

It is not obvious, yet possible, to see this thesis as an effectiveness analysis of PGD. Whereas the efficacy of PGD has been proven in early years by application of PGD-AS on a large scale (Donoso *et al.*, 2007), albeit with controversial outcome, the effectiveness of the technique in an unselected PGD/PGS population remains to be established. There are several reasons for this.

In the first place we are not dealing with a standard population of patients. The patients are even more inhomogeneous in baseline characteristics than a population undergoing routine IVF/ICSI, for example in view of the different modes of inheritance leading to a different embryo selection level, and the large proportion of patients not suffering with any fertility problems. Poor comparability of subjects on the other hand induces significant bias in outcome reporting in PGD, and the patient who will be informed about the reproductive prognosis on the basis of the analyses presented in this thesis, should be made aware of this.

Secondly, the outcome is not dichotomous in a sense that the result is not only the birth of a child or not, but the genetic health status of that child as well as the genetic status and affected rate of the embryos tested.

Thirdly, this technique cannot be studied in a randomised controlled way.

The right time scale criterion is difficult to determine in PGD. Whereas in conventional IVF/ICSI time to conception is the ideal standard, due to several factors this is difficult to apply in PGD. The time of intake is not usually the start of the treatment i.e. there is usually a significant time lag needed for establishing the details of the genetic condition and preparing the PGD markers and/or probes. This time period until the actual start of the PGD treatment varies according to the genetic condition tested for, and can vary from 2 to 24 months. The interval between different treatments is often shorter due to a higher embryo transfer cancellation rate and a higher degree of priority, especially with those disorders that require PGD associated with HLA matching. The optimum time scale criterion for PGD treatment analysis therefore seems *treatment cycle*.

Attempts have been made at designing more accurate statistical tools to assess cumulative reproductive outcome in reproductive medicine, including selective dropout exclusion (Stolwijk *et al.*, 1996; Land *et al.*, 1997) and multiple imputation (Soullier *et al.*, 2008).

Despite the limitations of life table analysis for PGD in terms of time scale criterion and informative censoring to some degree, as well as a number of modifications to diagnostic and therapeutic modalities over the years that are studied in this paper, subjects included towards the latter part of the study group are not likely to differ systematically from those who underwent PGD earlier in the study. However secular trends (Daya, 2005) are to some degree inevitable, due to changes in ovarian stimulation regimens (eg. the introduction of antagonist regimens as from 1996), the application of single versus double cell biopsy (Goossens *et al.*, 2008) and the changes in embryo transfer policy, but the changes have been relatively confined by the consistent genetic selection criteria and genetic diagnostic techniques, the limited number of ovarian stimulation protocols available and applied, as well as the consistent ICSI technique applied.

Nevertheless, and in spite of all pros and cons of life table analysis in reproductive medicine and in PGD more particularly, there are few alternatives to establish the cumulative reproductive prognosis other than calculating observed and expected delivery rates. The patient and partner should therefore be guided in their decision to embark on a treatment on an individual basis, taking into account all background characteristics including age, fertility status, parity, estimated or established ovarian response and the genetic condition they are treated for, and recognise that the expected cumulative delivery rate will overestimate, whereas the observed cumulative delivery rate will underestimate their prognosis. The latter will encourage clinics to be more realistic when counseling couples about prognosis and will discourage claims of treatment and clinic superiority (Daya, 2005). Comparison with other centres is difficult, mainly due to a different patient population, and reports such as the ESHRE Consortium reports should therefore be interpreted with caution (Goossens *et al.*, 2008).

Observational studies are suitable to detect rare or late adverse effects of treatment, and are more likely to provide an indication of what is achieved in daily medical practice. Other advantages include the opportunity to study in an unselected population, avoiding selection and therefore publication bias.

Results

Reproductive outcome of PGD

Over a period of 12 years, we assessed the outcome of 2753 ICSI-PGD cycles in 1498 couples. The main findings of this study were (i) that age and the number of oocytes significantly affect the reproductive outcome in PGD and (ii) that the rate of availability of genetically transferable embryos after PGD, fertility status and parity do not have a significant effect (table 2).

The cumulative observed delivery rate overall per couple with a maximum of 6 treatment cycles of ICSI and PGD performed was 29%. The expected cumulative delivery rate (max 6 cycles) overall was 62% (Fig. 3a and b). The effect of genetic selection of embryos is unfavourable as the overall expected cumulative delivery rate of 62% is much lower than the 79% overall expected cumulative delivery rate reported in a non-PGD ICSI population at our centre (Osmanogaoglu *et al.*, 1999). However, the mode of inheritance hence the rate of genetically transferable embryos did not have a significant effect as an independent factor on cumulative reproductive outcome. Although the delivery rate per oocyte collection cycle (OCC) is lower in some groups, particularly in the reciprocal translocations group, as is to be expected as a result of a higher rate of unbalanced and therefore untransferable embryos, the reproductive performance in terms of cumulative delivery rate was not significantly lower. The clinical pregnancy rate and delivery rate per ET are similar in all genetic subgroups, suggesting an isolated effect of genetic embryo selection especially in some subgroups such as the reciprocal translocations group where the availability of transferable embryos after PGD was low as 30%. The lack of a significant influence of the HLA typing group on the results per ET, as well as the lack of effect of number of oocytes in the HLA group, can presumably be attributed to underpowering.

The number of oocytes collected at retrieval significantly contributes to the cumulative reproductive outcome as an independent factor with an OR of 1.02, however ROC analysis reveals that the area under the curve (AUC) at 63% does not allow setting a threshold below which the prognosis is significantly impaired. This is in contrast with previous studies reporting a threshold level of 9 oocytes below which a significant reduction in reproductive outcome could be expected (Vandervorst *et al.*, 1998). Very few publications have addressed this issue in PGD. A recent study by Tur-Kaspa *et al.* showed that the availability of normal/unaffected embryos and the chances of pregnancy increase with increasing numbers of oocytes retrieved, but that a lower number of oocytes (less than 8) is still associated with a fair chance for normal/unaffected blastocyst transfer, especially in young patients, implying that routine canceling of cycles with an anticipated low number of oocytes is to be reconsidered (Tur-Kaspa *et al.*, 2008).

The fact that the fertility status did not contribute significantly to the cumulative reproductive prognosis is to be attributed to the bias effect of ICSI. Previous studies showed that the cumulative probability of an ongoing pregnancy was higher in women with secondary infertility than in those with primary infertility (Stolwijk *et al.*, 2000). This was not confirmed in our study, where multiparity did not have a significant independent effect on cumulative reproductive outcome of PGD, most probably due to the particular population with a low documented infertility ratio (35.3%). Consistent with reports in regular assisted reproductive treatment (ART) (Templeton *et al.*, 1996), advanced age of the female has a significant and independent negative effect on reproductive outcome, and those couples in which the female partner is aged 40 and over should be counseled very clearly on the very limited prognosis (Fig. 3a and b). Within the group over 39 years of age, there are no significant differences. Cumulative life table analysis

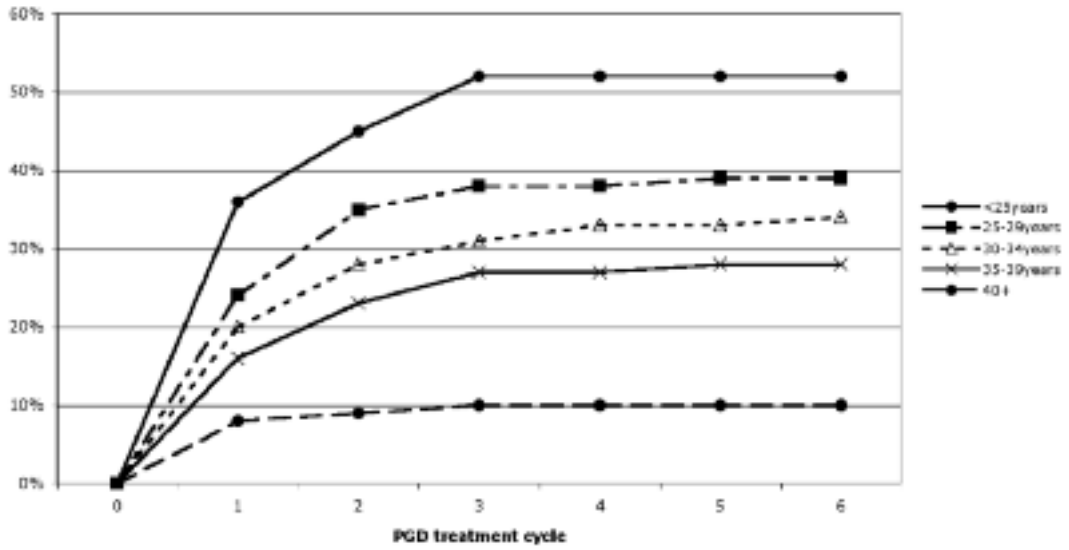

Fig. 3a. — Observed cumulative delivery rates of PGD in different age groups

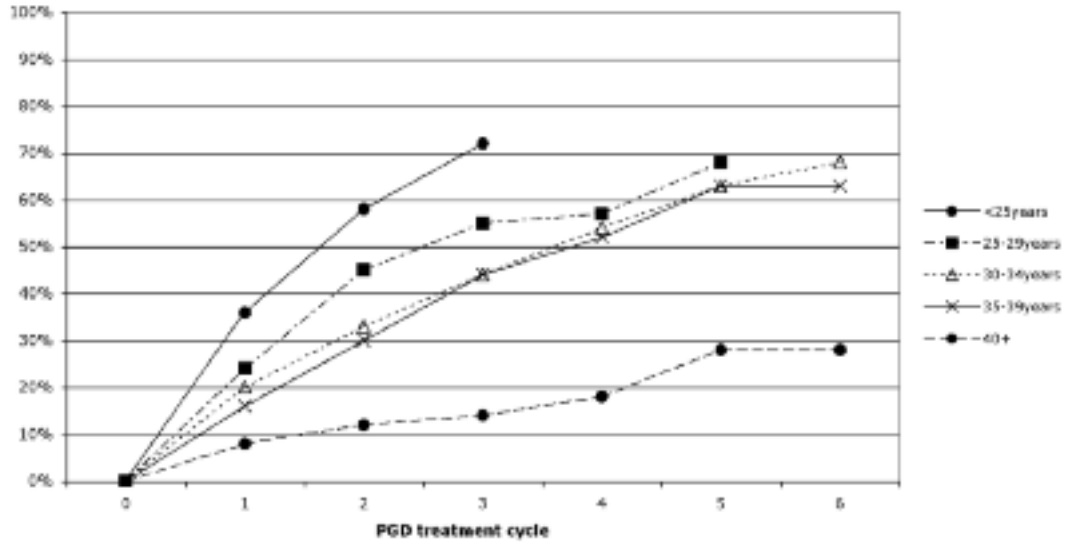

Fig. 3b. — Expected cumulative delivery rates related to age groups

is based on the assumption that dropout patients have the same probability as patients proceeding to a next attempt for IVF or ICSI after failed treatment. Previous studies have established the validity of life table analysis as a prognostic tool, in reproductive medicine (Hull, 1992; Osmanagaoglu *et al.*, 1999; De Vries *et al.*, 1999) even though the expected cumulative delivery rate potentially overestimates the true reproductive outcome over a large number of treatment cycles, due to informative censoring as well as a selection bias of good responders. In our study, the observation that the mean number of cycles per couple overall is 2.1 (SD 1.7) and the consequent finding that the observed cumulative delivery rate barely increases in treatment cycles 4 to 6 indicate that figures of cumulative reproductive outcome beyond 3 cycles should be interpreted with caution. In spite of a statistical increase in reproductive outcome in cycles 4 to 6 in all age groups, the clinical relevance is limited due to both informative and non-informative censoring. Although the data did not allow statistical comparison between genetic categories of the mean number of cycles performed, the latter does not seem differ in the Robertsonian or the reciprocal translocation group compared to the other groups, indicating that censoring did not occur more often in these indications. The same conclusions therefore apply with regard to clinical

relevance of cumulative outcome in higher order cycles for couples undergoing PGD for chromosomal translocations.

Previous studies have confirmed that there is no significant difference in background characteristics and/or the occurrence of prognostic indicators of poor treatment) of couples who continued treatment versus those who dropped out i.e. there is no informative censoring in the first two (Roest *et al.*, 1998) to three treatment cycles (Croucher *et al.*, 1998). The drop-out rate in our cohort was fairly constant at a mean of 48% (range 42-58%; not increasing) per treatment cycle, consistent with dropout rates of 23 to 45% for regular IVF/ICSI reported in literature (Gleicher *et al.*, 1996), and contrary to reports of dropout rates increasing with each successive cycle (Land *et al.*, 1997). We were not able to establish the reasons for dropout, but the mean number of cycles performed is similar to that reported in other studies (Osmanogaoglu *et al.*, 1999 (mean 1.91); Malizia *et al.*, 2009 (mean 2.3). The mean number of cycles performed in the HLA typing group of couples is remarkably higher at 2.6 (SD 1.6), indicating a higher level of compliance, in spite of limited success rates secondary to a highly reduced mean number of transferable embryos and a higher mean female partner age (Van De Velde *et al.*, 2008).

Results of cryopreservation in the PGD program

The cryopreservation and thawing results of blastocysts at our centre, as well as the reproductive outcome of the cryopreservation/thawing program over the years studied are low. We only considered blastocyst culture for this study because this was the exclusive type of culture since 2001 for PGD embryos. Between 2001 and 2005, 2252 cycles of PGD/PGS were performed, versus 1970 cycles of conventional ICSI with blastocyst culture. Compared to blastocyst cryopreservation in conventional ICSI, the cryopreservation rate per PGD/PGS cycle was lower (19.9 vs 58.6%; $p < 0.01$), whereas the transfer rate following thawing was not significantly different (52.1% vs 42.7%; $p = 0.06$). The delivery rate per thawing cycle was similar in both groups (4.2% vs 4.8%; $p = 0.79$), as was the delivery rate per transfer (8.1% vs 11.2%; $p = 0.47$). The frozen/thawed blastocyst implantation rate was comparable in the PGD group compared to the control group (5.6% vs 7.7%; $p = 0.67$) (table 3).

Moreover, the fact that the LBR per ET is nearly double the LBR per thawing cycle, indicates the poor survival rate of the blastocysts in the cryoprogram 2001-2005. Because of the low reproductive outcome, it is impossible to evaluate the impact of PGD on reproductive outcome using frozen/thawed blastocysts. Observing a reduction of ~30% (from 11.2 to 8.1% (table 3)), which is in general accepted as clinically relevant, 3832 control cases and 958 PGD cases would have been necessary to understand the significance of this observation. For implantation rate per transfer, a difference of ~30% (from 7.7% to 5.6% (table 3) requires the inclusion of 5940 blastocysts in the control group and 1485 in the PGD group. With this level of success it is practically impossible to demonstrate a difference between the 2 groups (Verpoest *et al.*, unpublished data).

Risks associated with PGD

Misdiagnosis

Prenatal diagnosis following preimplantation genetic diagnosis or screening is a delicate issue. Taking into account that up to 29% of couples apply for PGD on the basis that they object to termination of pregnancy (Harper *et al.*, 2006), and knowing that the risk of total fetal loss following CVS and AC is reported to be as high as 2.0% and 1.9% respectively (Mujezinovic and Alfirevic, 2007), a significant number of couples (57.5%; Harper *et al.*, 2006) are not keen to undergo invasive prenatal diagnosis. Advice to undergo invasive prenatal diagnosis is given on the basis of a potentially false negative result of around 0.83% (based on ESHRE consortium data I-VIII; 24 reported misdiagnoses/ 2885 ongoing pregnancies >12 weeks; Goossens *et al.*, 2008).

In the cohort of couples undergoing PGD 1993-2005, and more specifically of the 314 PGD foetuses and 272 PGS foetuses respectively that were conceived as a result of this treatment, 138 (44%) and 34 (11%) were tested prenatally by CVS or amniocentesis. Postnatally 32 PGD children were tested for the at risk condition. In 56 of the PGS children a karyotype was performed and 237 of them had a normal physical examination. In the PGD group 4 misdiagnoses were seen in 170 tested foetuses/children. One occurred in a case at risk for myotonic dystrophy (Sermon *et al.*, 2001) and 3 occurred in the one couple at risk for CMT 1A. The latter misdiagnosis was the result of an erroneous linkage analysis prior to PGD resulting in the selection of affected embryos for transfer (Goossens *et al.*, 2008). The misdiagnosis rate is therefore either to be quoted as 1/172 (0.6%) when only taking into account the true misdiagnosis for DM1. When taking account the erroneous linkage analyse i.e. wrong pre-PGD work-up, the misdiagnosis rate is 4/172 (2.3%) (Liebaers *et al.*, 2009).

Antepartum care in ART pregnancies following PGS or PGD should therefore include counseling couples on the remaining minimised risk of a genetic abnormality, the risks of invasive prenatal testing and

Table 3. — Contribution of factors to cumulative reproductive outcome based on logistic regression modeling.

	OR (95% CI) unadjusted odds ratio	OR (95% CI) adjusted odds ratio*
age		
< 25 years reference category	–	–
25-29 years	0.86 (0.42-1.72)	0.92 (0.45-1.88)
30-34 years	0.60 (0.34-1.49)	0.61 (0.30-1.24)
35-39 years	0.56 (0.28-1.11)	0.53 (0.26-1.07)
> 40 years	0.25 (0.12-0.55)	0.22 (0.10-0.50)
number of oocytes	1.02 (1.01-1.04)	1.02 (1.00-1.04)
genetic category		
50% reference category	–	–
75% available embryos for transfer	1.20 (0.87-1.65)	0.90 (0.54-1.49)
robertsonian translocations	1.39 (0.87-2.22)	1.24 (0.70-2.20)
reciprocal translocations	0.72 (0.46-1.14)	0.75 (0.43-1.33)
HLA typing	0.56 (0.21-1.51)	0.73 (0.26-2.05)
other chromosomal abnormalities	1.11 (0.67-1.86)	1.13 (0.55-2.33)
PGS	0.98 (0.78-1.25)	1.36 (0.97-1.91)
fertility status	1.04 (0.85-1.27)	1.00 (0.77-1.29)
parity	0.93 (0.71-1.20)	1.09 (0.83-1.44)
mode of pituitary suppression	0.93 (0.76-1.14)	1.07 (0.84-1.36)

* odds ratio simultaneously adjusted for all other variables listed in the first column of Table 3.

the risks associated with a genetic disorder if and when present in the pregnant patient.

Perinatal outcome of PGD

The main finding of this prospective comparative data analysis is that embryo biopsy and extended culture for PGD does not increase the short-term risk of major congenital abnormalities when compared to a historic control population of children conceived by regular ICSI without PGD, and when compared to other publications on major malformations following PGD (major malformation rate 0.9% in 102 PGD children (Strom *et al.*, 2000a,b) and 2.2 to 4.3% on 2286 children calculated from the publications of and the ESHRE PGD consortium (Goossens *et al.*, 2008)). The perinatal death rate is particularly high for in PGD multiple pregnancies when compared to the control group of multiples conceived by ICSI, albeit without a clearly identifiable reason. The fact that the perinatal mortality in multiples almost exclusively occurs in very premature multiples, in association with the multiple pregnancy rate of 19.7% is a strong reason for imposing more stringent guidelines of restricting the number of embryos for transfer (Liebaers *et al.*, 2009).

Monozygotic twinning

The incidence of predominantly bichorionic twins in ART is evidently increased as a result of the transfer of multiple embryos in a lot of cases. The incidence of MZ twins however has also been reported to be increased two to four-fold in ART (Cohen *et al.*, 1992; Costa *et al.*, 2001). MZ twinning has previously been shown to be unrelated with maternal age, paternal age, gonadotrophin dosage, peak estradiol and progesterone levels, number of oocytes collected and number of embryos transferred (Alikani *et al.*, 2003). Some have argued a causal relationship between the higher number of embryos transferred and MZ twinning (Scott Sills *et al.*, 2000). Possible mechanisms include breaks in the zona pellucida associated with handling, and especially the creation of intentional holes e.g. by ICSI or embryo biopsy. PGD is performed on a single or dual blastomere extracted from the embryo at cleavage stage, following the creation of a small hole in the zona pellucida by laser. The technique for breaching the zona pellucida by laser in order to perform embryo biopsy for PGD is to some degree analogous with that used for assisted hatching (AH).

Embryo biopsy for PGD does not cause an increase in the incidence of MZ twins. This information is useful in counseling couples undergoing PGD about the potential risks of the technique. MZ twins are at high risk of perinatal morbidity and mortality in view of the low mean birth weight and low gestational age at birth, also observed in this study. Larger sample sizes are required to provide higher statistical power (Verpoest *et al.*, 2009).

Table 4a. — Baseline and outcome characteristics per oocyte collection cycle (OCC).

	PGD group 2001-2005	control group 2001-2005	p-values
mean age	34.4 (SD 4.9)	32.2 (SD 4.8)	**p < 0.01**
no. of oocyte collection cycles (OCC)	2252	1970	
no. of transfer cycles in OCC	1596	1794	
transfer rate (% of OCC)	70.9%	91.1%	**p < 0.01**
delivery rate per OCC	17.6%	29.6%	**p < 0.01**
delivery rate per ET	24.9%	32.6%	**p < 0.01**
number of deliveries	397	584	
number of children born	473	712	
no. of cycles with cryopreservation	318	1051	
cryopreservation rate per transfer cycle (%)	19.9%	58.6%	**p < 0.01**
no. of embryos frozen	811	3559	
mean no. of embryos frozen	2.6 (SD 2.3)	3.4 (SD 2.4)	**p < 0.01**

Table 4b. — Baseline and outcome characteristics per thawing cycle.

no. of thawing cycles	119	583	
no. of embryos thawed	383	2231	
mean no. of embryos thawed	3.3 (SD 2.1)	3.8 (SD 2.1)	**p < 0.05**
no. of frozen/thawed embryo transfer procedures	62	249	
(% per thawing cycle)	52.1%	42.7%	p = 0.06
no. of embryos transferred	90	414	
mean no. of embryos transferred	1.5 (SD 0.6)	1.7 (SD 0.6)	**p < 0.05**
no. of positive hCG measurements	9	53	
no. of deliveries	5	28	
(% of thawing cycles)	4.2%	4.8%	p = 0.79
(% of transfer cycles)	8.1%	11.2%	p = 0.47
singleton	5	24	
twin	0	4	
triplet	0	0	
implantation rate (%)	5.6%	7.7%	p = 0.67
no. of children born	5	32	

statistically significant p-values are indicated in bold.

Conclusion

With increasing numbers of PGD performed, as well as expanding diagnostic possibilities and rapidly growing numbers of children born following PGD, accurate outcome analysis is required, in order to correctly counsel patients and their partners, and guide them in their decision whether or not to proceed to PGD treatment. On the basis of the analyses performed that have led to this thesis, we can conclude that the overall true chance of delivering at least one child per couple undergoing PGD is 29%, if a maximum of 6 treatment cycles is accepted, and that this is an underestimate based on non-informative censoring of certain couples. The expected delivery rate for these couples based on a Kaplan-Meier calculation is overall 62%, which is an overestimate based on inclusion of poor prognosis couples who would have been censored prior to completing 6 full treatment cycles (informative censoring). The age of the female partner and the number of oocytes collected each have a significant and independent effect on the cumulative delivery rate. The reproductive prognosis of PGD is significantly worse in couples in which the female partner is over 40 years of age compared to younger age groups. Parity, fertility status, mode of pituitary suppression and the category of genetic inheritance expressed as the degree of genetically normal embryos available for embryo transfer did not have a significant independent effect on cumulative delivery rate, according to the statistical analyses performed in the study cohort.

A number of risks associated with PGD practice can be observed. The risk of misdiagnosis is very limited to less than 1% in the study cohort, however on the basis of potential technical and human errors, as well as on the risk of mosaicism in embryos biopsied and tested, patients can be advised to undergo invasive prenatal diagnosis by CVS or AC. The

urgency of such a prenatal diagnosis needs to be assessed on an individual basis and based on the couple's background characteristics including age of the female partner, the diagnostic accuracy of the diagnostic test and the psychological status of the patient.

The risk of perinatal death in infants born after PGD is increased compared to a control population undergoing ICSI, largely due to a large number of very prematurely born multiples born under 25 weeks of gestation in the PGD cohort. This is again a strong call for a limitation of the number of embryos transferred, optimisation of cumulative outcome by means of improved cryopreservation programs, and close follow up in pregnancy by experienced obstetricians. The risk of monozygotic twinning is 1.5%, and is not increased after PGD compared to ICSI with blastocyst transfer according to the analyses performed in the study cohort.

Design or too much design: the clinical information provided in this thesis should guide both patients and physicians in their decision to proceed to PGD.

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