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Successful preimplantation genetic diagnosis is related to the number of available cumulus–oocyte complexes

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The inheritance pattern of monogenic inheritable disorders influences the proportion of unaffected embryos after preimplantation genetic diagnosis (PGD). We aimed to investigate the influence of the number of cumulus–oocyte complexes (COC) on the outcome after PGD. Eighty-four cycles of 47 couples were included in our analysis. All couples were at risk of transmitting autosomal recessive, autosomal dominant, X-linked single gene disorders or sex-aneuploidies to their offspring. One PGD cycle was carried out for a Y_q-deletion of the man. The correlation between the numbers of COC and biopsied embryos and between the numbers of COC and unaffected embryos was highly significant (P <0.05). A pregnancy occurred in 15 cycles and a minimum of six COC were needed to achieve a pregnancy. Thirteen pregnancies were observed in cycles with at least 9 COC. The transfer rate and number of transferred embryos per cycle in the subgroups with <9 COC and ⩾9 COC were significantly higher in the latter. Although pregnancy rates did not differ significantly between the two subgroups (probably due to the low number of pregnancies), our data indicate that it is justifiable to cancel PGD cycles in which it is expected that <6 COC will be retrieved and that the couple should be informed about the poor prognosis if <9 COC are retrieved.

Key words: embryo biopsy/number of cumulus–oocyte complexes/ovarian stimulation/preimplantation genetic diagnosis

Introduction

Preimplantation genetic diagnosis (PGD) has been developed to prevent the transmission of genetic diseases to offspring. PGD may be seen as an alternative to prenatal diagnosis (PND), which has been used successfully for the same purpose. PGD differs from conventional PND in that in the former embryos are biopsied and genetically tested at a very early cleavage stage before implantation, while in the latter the genetic diagnosis is carried out during pregnancy in the fetal (chorionic villus sampling or amniocentesis) stage. In order to obtain PGD-embryos, couples first have to undergo ovarian stimulation followed by assisted reproductive treatment. Subsequently, only genetically non-affected embryos are transferred into the uterus. In this way, PGD avoids the emotional distress associated with termination of pregnancy at a more advanced gestational age in cases where an affected fetus is diagnosed.

The risk of transmitting a genetic disorder differs according to the inheritance pattern of the disease. Among male offspring of women carrying a recessive X-linked disorder, 50% are affected. When the father carries the Y_q-deletion, this will be transmitted to all his male offspring. When both parents are heterozygous for an autosomal recessive disorder, the risk of a homozygous affected child is 25%. In cases where one of the parents is affected by an autosomal dominant disease, 50% of the offspring will also be affected. Theoretically, one-quarter to one-half of all embryos obtained in PGD cycles will be affected by the disease under consideration and consequently they will not be suitable for transfer. The situation is different in patients with Klinefelter's syndrome, since the risk of sex-chromosome aneuploidy in the offspring is not known. Furthermore, the number of available embryos in these patients depends strongly on the number of available spermatozoa in the ejaculate or testicular biopsy specimens.

Since the introduction of intracytoplasmic sperm injection (ICSI) into the field of reproductive medicine, couples suffering from severe male infertility may conceive. The outcome of ICSI is not related to sperm density, motility, morphology or origin (Nagy *et al.*, 1995a,b). Other authors have reported higher rates of pregnancy loss after the transfer of embryos obtained by testicular or epididymal spermatozoa compared with ejaculated spermatozoa (Tournaye *et al.*, 1994; Palermo *et al.*, 1995; Kahraman *et al.*, 1996). We have demonstrated previously that the numbers of cumulus–oocyte complexes (COC) and ongoing pregnancy rates are strongly correlated (Vandervorst *et al.*, 1997). It is clear from ICSI cycles without PGD that the number of retrieved COC is of more importance than sperm quality to predict the outcome of the cycle.

A major proportion of the couples who can benefit from PGD do not suffer from infertility. We therefore expect high two-pronuclear (2-PN) fertilization and normal embryo cleavage in these couples, but the number of oocytes may limit the number of transferable embryos. The number of COC will indeed affect the number of embryos that can be biopsied and diagnosed. Since 25–50% of the obtained embryos will be affected with the disease under consideration, and since the genetic analysis will fail in some embryos, an embryo transfer can only be carried out when a high number of embryos is available from the start.

The aim of our study was to relate the outcome of the

PGD cycles to ovarian responsiveness to ovarian stimulation, expressed as the number of retrieved COC. Furthermore, some guidelines for ovarian stimulation in PGD cycles will be given. In addition we will define a threshold number of COC for which it is meaningful to perform ICSI, embryo biopsy and genetic analysis.

Materials and methods

Patient population

Between February 1992 and July 1997, 54 couples at risk of transmitting a monogenic disorder or sex aneuploidy were counselled at our Centres for Reproductive Medicine and for Medical Genetics in view of PGD treatment. The couples were counselled for: Steinert's myotonic dystrophy, Marfan's syndrome, cystic fibrosis, β-thalassaemia, Duchenne's muscular dystrophy, X-linked mental retardation, haemophilia A, retinitis pigmentosa and Y_q-deletion. For specific reasons, a number of Klinefelter's syndrome patients were also offered PGD after oocyte fertilization by ICSI with testicular spermatozoa. In one case in which ICSI was necessary because of a severe male factor and in which the wife had a 47,XXX karyotype, PGD was also performed. Previously, some of these couples had conceived spontaneously. Some of these spontaneous pregnancies had been terminated in the first trimester after a PND had revealed an affected fetus, or had ended in the delivery of an affected child. Before PGD, female patients underwent a minor subfertility work-up, including basal serum follicle stimulating hormone (FSH) concentrations and transvaginal pelvic ultrasound, while every male partner had a semen analysis.

Ovarian stimulation

Ovarian stimulation was carried out by pituitary desensitization with gonadotrophin-releasing-hormone analogues (GnRHa) (buserelin; Suprefact®, Hoechst, Brussels, Belgium) combined with human menopausal gonadotrophins (HMG) (Humegon®; Organon, Oss, The Netherlands or Pergonal®; Serono, Brussels, Belgium). This protocol has previously been described in detail by Smitz *et al.* (1988). When the ovarian response in consecutive treatment cycles was not adequate, the patient was stimulated in a subsequent cycle with clomiphene citrate in a dose of 100 mg daily from day 3 to day 7 of her cycle, followed by the administration of at least 225 IU HMG daily from day 7 onwards. Human chorionic gonadotrophin (HCG) (10 000 IU; Pregnyl®, Organon or Profasi®, Serono) was administered when at least three follicles of 17 mm diameter were seen on vaginal ultrasound scan. Transvaginal ultrasound-guided oocyte retrieval was scheduled 36 h after HCG administration. The luteal phase was supplemented by 600 mg micronized progesterone daily, administered intravaginally (Utrogestan; Piette, Brussels, Belgium). Regular in-vitro fertilization (IVF) was carried out in four cycles, while in all the other cycles microinjection was used for reasons of male subfertility or for technical reasons related to the genetic analysis. The different steps of the ICSI procedure and the preparation of the injection and holding pipettes have been described previously by our group (Van Steirteghem *et al.*, 1993, 1995). ICSI was carried out with ejaculated spermatozoa, while in cases of azoospermia, epididymal or testicular spermatozoa were used. Epididymal spermatozoa were retrieved by microsurgical epididymal sperm aspiration (MESA) (Silber *et al.*, 1994). Testicular spermatozoa were obtained by testicular excisional biopsy, a technique previously described by Devroey *et al.* (1995).

At 16–18 h after the injection procedure, all oocytes were evaluated for intactness and fertilization (Nagy *et al.*, 1994); the quality of the embryos was assessed one day later. According to the number of anucleate fragments, the embryos were subdivided into grades A (no anucleate fragments), B (1–20% anucleate fragments), C (21–50% anucleate fragments) and D (>50% anucleate fragments) embryos. In the morning of day 3, grade A, B and C embryos were biopsied. Two blastomeres were removed from those embryos which contained seven or more blastomeres. When the embryo held less than seven blastomeres, only one blastomere was removed. Embryo biopsy was accomplished by making a hole in the zona pellucida by a stream of acidic Tyrode with a fine needle. The blastomeres were removed from the embryo by gentle aspiration through the hole. When embryonic compaction had already started the embryos were incubated in Ca^{2+}- and Mg^{2+}-free medium before the biopsy (Sermon *et al.*, 1997). The genetic diagnosis was carried out using either the polymerase chain reaction (PCR) or fluorescence in-situ hybridization (FISH) techniques (Lissens and Sermon, 1997). Whenever possible, PCR was carried out to detect the single gene defect. A specific PCR was used for cystic fibrosis (Liu *et al.*, 1994a,b), Steinert's myotonic dystrophy (Sermon *et al.*, 1997), Marfan's syndrome (Pereira *et al.*, 1994), β-thalassaemia (Ray *et al.*, 1996; Van de Velde *et al.*, 1997) and Duchenne's muscular dystrophy (Liu *et al.*, 1995). Sex determination of embryos in cases of X-linked diseases and sex-chromosome aneuploidies (Klinefelter's syndrome) was done by FISH (Coonen *et al.*, 1994; Griffin *et al.*, 1994; Munné *et al.*, 1994; Staessen *et al.*, 1996). Fresh day 3 embryos were transferred immediately after the genetic analysis was carried out. Between the time of embryo biopsy and the time of embryo transfer, embryos were kept in culture. When the couple was tested for cystic fibrosis or β-thalassaemia (autosomal recessive), the best homozygous and heterozygous embryos were transferred. When the male and female cystic fibrosis mutation differed, the embryos were analysed for only one mutation. Only embryos which did not carry the tested mutation were accepted for transfer. Exclusively homozygous normal embryos were transferred in cases where the couple was tested for a dominant single gene defect. Only female embryos were transferred when sexing was used in embryos at risk of an X-linked disorder. As in cycles without PGD, the age of the patient, the rank of trial and embryo quality (whenever the embryo transfer was elective) determined the number of embryos to be transferred (Staessen *et al.*, 1993, 1995). If supernumerary unaffected embryos with <50% fragmentation continued to cleave between the time of biopsy and the time of freezing, i.e. the evening of day 3 or the morning of day 4 post insemination, they were cryopreserved (Van Steirteghem *et al.*, 1994). Affected embryos or embryos with >50% fragmentation were further analysed in order to confirm and improve the test procedure.

Implantation was confirmed when two serum HCG concentrations at least 10 days after embryo transfer showed a gradual increase. A clinical pregnancy was noted when an intrauterine gestational sac was seen on vaginal ultrasound at least 5 weeks after the embryo replacement. An ongoing pregnancy was defined as a clinical pregnancy with a fetal heart beat >12 weeks. In these cases, the couple was recommended to undergo a prenatal diagnosis (chorionic villus sampling or amniocentesis) in order to confirm the PGD diagnosis.

Statistical analysis

Two-tailed Student's *t*-test and chi-squared test were used respectively to compare mean values and proportions at the 5% level of significance. We made use of a parabolic regression model ($y = a + bx + cx^2$) to relate the numbers of retrieved COC to the PGD outcome.

Results

Although 54 couples were enrolled in the PGD programme, only 47 completed the entire PGD procedure. Between Febru-

Table I. Number of patients and preimplantation genetic diagnosis (PGD) cycles according to the origin of the genetic disorder and analysis technique (PCR or FISH) used

Indication	Patients (*n*)	Cycles (*n*)
PCR		
Monogenic diseases		
Myotonic dystrophy	13	31
Cystic fibrosis	10	23
Duchenne's muscular dystrophy	3	5
Marfan's syndrome	1	1
β-Thalassaemia	1	1
FISH		
Monogenic diseases (*sexing*)		
Haemophilia A	3	4
X-linked mental retardation	3	5
Retinitis pigmentosa	1	1
Duchenne's muscular dystrophy	3	3
Structural chromosomal aberration (*sexing*)		
Y_q-deletion	1	1
Sex-aneuploidy screening		
Klinefelter's syndrome	7	7
47,XXX[a]	1	2
Total	47	84

[a]PGD was carried out since intracytoplasmic sperm injection was necessary for a severe andrological problem.
PCR = polymerase chain reaction; FISH = fluorescence in-situ hybridization.

Table II. Influence of the inheritance pattern of the tested disease on the number of unaffected embryos and on the number of transferred and cryopreserved embryos

Inheritance pattern	Monogenic recessive	Monogenic dominant	Monogenic X-linked -Y_q-deletion	Sex-aneuploidy
Cycles (*n*)	29	33	14	8
COC (*n*)	405	398	221	116
Biopsied embryos (*n*)	133	202	111	40
Unaffected embryos (*n*)	84	63	35	18
Unaffected embryos				
Per COC	0.21	0.16	0.16	0.15
Per biopsied embryo	0.63[a,b]	0.31[a,c]	0.31[b,d]	0.45[c,d]
CEI	0.19	0.14	0.13	0.20

[a,b]$P < 0.00005$.
[c]$P < 0.005$.
[d]$P < 0.05$.
Values with the same superscript were significantly different.
CEI = cycle efficiency index; COC = cumulus–oocyte complex.

Table III. Influence of the number of transferred embryos on pregnancy rates

	No. of transferred embryos				
	0	1	2	3	4[a]
No. of cycles	16	22	24	21	1
No. of pregnancies (%)	0 (0)	2 (10.1)	5 (18.1)	7 (33.3)	1 (100)

[a]Four embryos were transferred in this patient because of bad embryo cleavage and bad embryo morphology.

ary 1992 and July 1997, these 47 couples underwent 84 cycles, the number of cycles varying between one and five attempts. Two couples started a subsequent PGD cycle after a successful previous one. The mean age of the female patients was 31.4 (range 24–38) years.

Regular IVF was carried out with ejaculated spermatozoa in two cycles for cystic fibrosis in which the male partner presented normal vasa deferentia, in one cycle for X-linked mental retardation, and in one cycle for muscular dystrophy. In the other cycles, ICSI was carried out. In the group of patients undergoing PGD for cystic fibrosis, four males presented congenital bilateral absence of the vas deferens (CBAVD). In these four couples, six ICSI cycles were carried out with fresh epididymal spermatozoa. In respectively one and five subsequent cycles of these couples, testicular spermatozoa and frozen–thawed spermatozoa were used for microinjection. In another couple, who underwent PGD for myotonic dystrophy, the male partner suffered from non-obstructive azoospermia. In this case, enough motile spermatozoa were retrieved after testicular sperm extraction (TESE) to inject all MII-oocytes of his wife. Fourteen Klinefelter's syndrome patients (16 cycles) underwent a TESE procedure. In only seven patients (eight cycles) were sufficient numbers of testicular spermatozoa retrieved to inject all oocytes. Only these seven couples were included in the study. The number of patients and number of cycles per tested disorder in which PGD was carried out are listed in Table I.

A total number of 1140 COC were retrieved, i.e. a mean of 13.6 (range 2–43) COC per cycle. Figure 1 shows the distribution of the number of cycles according to the number of retrieved COC. In total, 881 MII oocytes were microinjected, while regular IVF was performed on 79 oocytes. After ICSI and regular IVF, 2-PN fertilization ensued in respectively 610 and 49 oocytes (69.2% of the injected oocytes and 62.0% of the inseminated oocytes). A total of 600 embryos or 91% of the 2-PN oocytes reached a day 3 cleavage state. Since 486 embryos contained more than four blastomeres at 3 days after insemination, only these were biopsied. Seventy-seven cycles reached the biopsy phase, i.e. a biopsy rate of 92% per cycle. In 70 embryos we could not obtain a diagnosis due to blastomere lysis or non-amplification, while 216 embryos turned out to be affected by the disease under consideration (14.4% and 44.4% of the total number of biopsied embryos respectively). In total, 180 embryos were diagnosed as homozygous normal, while 20 embryos were healthy carriers (37% and 4.1% of the total number of biopsied embryos respectively), giving a total of 200 non-affected embryos. In addition, 137 embryos were transferred in 71 cycles, i.e. a mean number of 1.93 (range 1–4) embryos per transfer or 0.85 (range 0–4) embryos per cycle. Forty-eight embryos were cryopreserved. To estimate the influence of the disease under consideration, several subgroups were studied: recessive autosomal disorders, dominant autosomal disorders, recessive X-linked disorders (sexing) and sex-aneuploidies. The results of the PGD cycles according to the disease tested are given in Table II. In general, a clinical pregnancy occurred in 15 cycles. An association between pregnancy rates and the number of transferred embryos was observed (Table III). One pregnancy ended in a miscarriage, four pregnancies (including one twin pregnancy) were

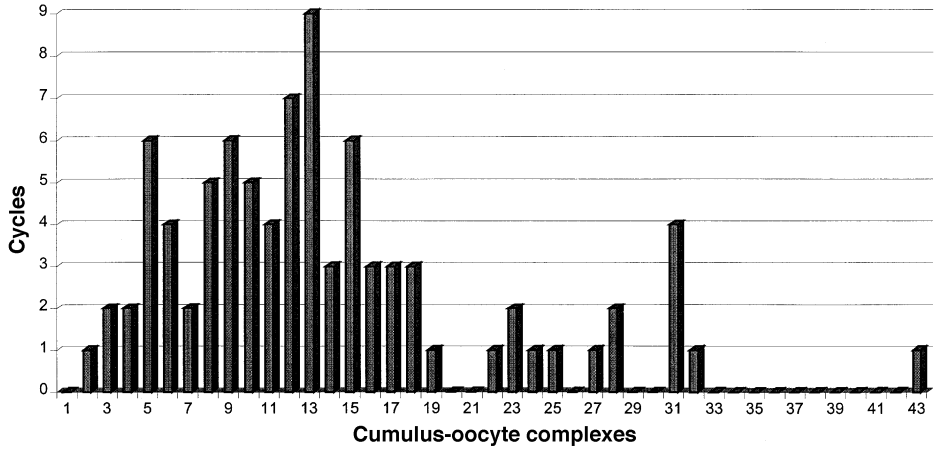

Figure 1. Distribution of the number of preimplantation genetic diagnosis cycles according to the number of retrieved cumulus–oocyte complexes.

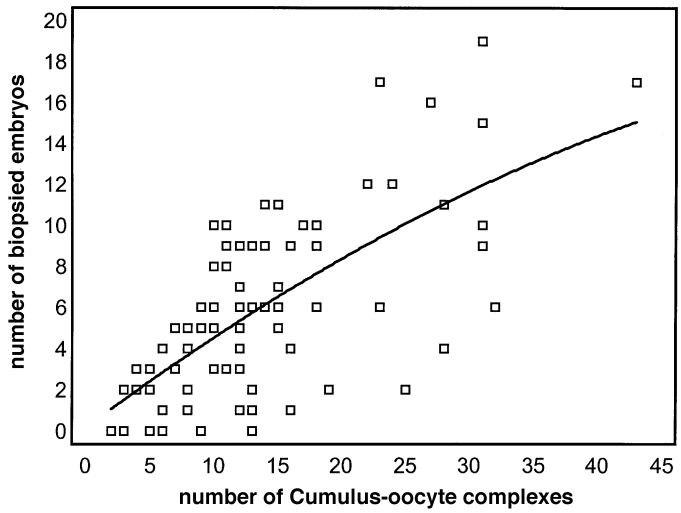

Figure 2. Parabolic regression analysis between the number of retrieved cumulus–oocyte complexes (COC) and the number of biopsied embryos. No. of biopsied embryos = 0.1084 + 0.4680 COC – 0.0028 $(COC)^2$; $P < 0.0005$.

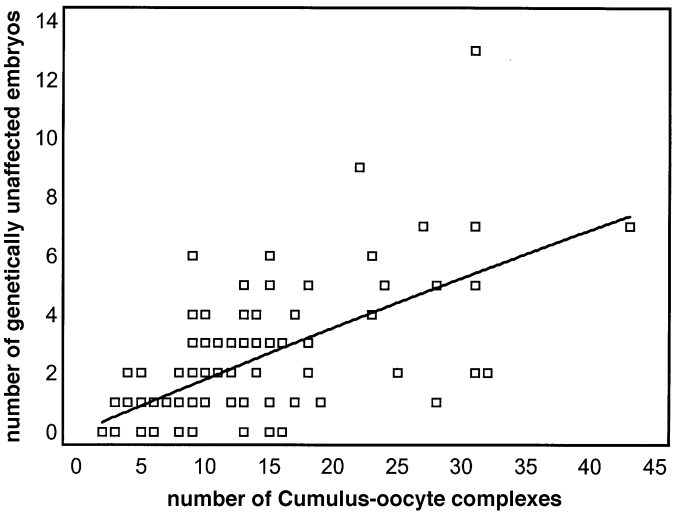

Figure 3. Parabolic regression analysis between the number of retrieved cumulus–oocyte complexes (COC) and the number of genetically unaffected embryos. No. of healthy embryos = –0.0467 + 0.1844 COC – 0.0003 $(COC)^2$; $P < 0.0005$.

still ongoing at the time of writing, and 13 children were born from 10 deliveries (eight singletons, one twin and one triplet). The triplet pregnancy occurred after monozygotic division of one embryo of a dizygotic twin pregnancy in a PGD cycle for Duchenne's muscular dystrophy. The singleton fetus developed normally, while the monozygotic twin consisted of one normal fetus and one acardiac fetus. After a premature delivery only the singleton fetus survived. Three frozen–thawed embryo transfers were planned, but in only two cycles were embryos available after thawing. No pregnancies occurred after transfer of frozen–thawed embryos. In all cycles, PGD results were confirmed by chorionic villus sampling, amniocentesis or after birth.

The relationship between the number of COC and the number of biopsied embryos is shown in Figure 2. A highly significant regression was observed ($y = 0.1084 + 0.4680x - 0.0028x^2$; $P < 0.0005$). A similar highly significant regression was demonstrated when the number of genetically unaffected embryos (homozygous and heterozygous normal) after successful analysis was plotted against the number of COC (Figure 3; $y = -0.0467 + 0.1844x - 0.0003x^2$; $P < 0.0005$). A mean number of 14.2 ± 8.03 COC were retrieved in cycles, which resulted in the biopsy of at least one embryo compared with 7.3 ± 4.5 COC in cycles in which no embryo biopsy could be done, a difference which was statistically significant ($P = 0.03$). When cycles resulting in embryo transfer were compared with those without embryo transfer, we retrieved a mean number of 14.6 ± 8.26 and 9.3 ± 4.57 COC ($P = 0.02$) respectively. Although the numbers of retrieved COC in the cycles which resulted in a pregnancy and those in which no pregnancy was achieved did not differ significantly (15.9 ± 8.3 in the pregnant subgroup and 13.1 ± 7.87 in the non-pregnant subgroup), no pregnancies ensued when <6 COC were retrieved. Thirteen of the 15 pregnancies (87%) occurred in cycles in which at least 9 COC were retrieved. The differences in the outcome parameters when <9 COC or ⩾9 COC were retrieved are shown in Table IV.

Table IV. Cycle characteristics when <9 and ⩾9 cumulus–oocyte complexes (COC) were retrieved

Cycle characteristics	No. of COC	
	<9	⩾9
No. of cycles (% of total)	22[a] (26.1)	62[a] (73.9)
Total no. of retrieved COC	124	1020
No. of cycles with embryo biopsy (%)	18 (82)	59 (95)
No. of biopsied embryos (mean ± SD)	2.23 ± 1.57[b]	7 ± 4[b]
No. of biopsied embryos/COC	0.40	0.43
No. of cycles with embryo transfer (%)	14[c] (63.6)	54[c] (87)
No. of healthy embryos/COC	0.14	0.18
No. of transferred embryos/cycle (mean ± SD)	0.77 ± 0.69[d]	1.94 ± 1.05[d]
No. of embryos/transfer (mean ± SD)	1.21 ± 0.68[e]	2.22 ± 1.05[e]
Implantation rate/embryo (%)	20	13
Pregnancy rate/cycle (%)	2/22 (9)	13/62 (20.9)

[a,b,d,e]$P < 0.0005$.
[c]$P < 0.05$.
Values with the same superscript were significantly different.
COC = cumulus–oocyte complex.

Discussion

Preimplantation genetic diagnosis (PGD) is a novel technique developed to prevent the transmission of monogenic diseases or sex aneuploidies. Since only healthy embryos obtained after assisted reproduction treatment are transferred, the emotional and psychosocial discomfort associated with induced abortion or termination of pregnancy at a more advanced gestational age when an affected fetus is diagnosed by PND are overcome. The need for apparently fertile couples to undergo ovarian stimulation and assisted reproduction treatment, with the associated emotional and physical distress, may be a drawback to this technique. Gamete donation and adoption—alternatives to PGD and PND—can also be considered in these couples. Snowdon and Green (1997) have demonstrated in couples carrying a recessive disorder, that both PGD and PND were preferred above these alternatives.

An association between the number of fertilizable eggs and pregnancy rates after regular IVF and ICSI has been established (Arnot *et al.*, 1995; Sherins *et al.*, 1995; Roest *et al.*, 1996; Meniru and Craft, 1997). In a previous retrospective analysis, performed on 4697 ICSI cycles, we demonstrated that ongoing pregnancy rates increased significantly when at least 7 COC were obtained after ovarian stimulation, as compared with the cycles with <7 COC (Vandervorst *et al.*, 1997). Since the influence of the sperm characteristics on the ICSI process is minimal (Nagy *et al.*, 1995a,b), except for an increased risk of embryo wastage when non-ejaculated spermatozoa are used (Tournaye *et al.*, 1994; Palermo *et al.*, 1995; Kahraman *et al.*, 1996), the number of COC may become an extremely important predictive tool for the outcome, once spermatozoa are available for injection.

To our knowledge, this is the first report which relates responsiveness to ovarian stimulation with the outcome of PGD cycles. We demonstrated that the need for a reasonable number of fertilizable eggs was even more important in PGD cycles than in regular ART cycles. More than 35% of the biopsied embryos in PGD cycles for monogenic recessive disorders were genetically affected. In PGD cycles for monogenic dominant and X-linked disorders the proportion of affected embryos rose to 70%. We showed clearly that the success rate of PGD was associated with the retrieval of at least 9 COC and demonstrated by regression analysis that a higher number of COC was associated with a higher number of embryos for biopsy. In turn, this resulted in a significantly higher number of embryos not affected by the disease at risk, with consequently, a higher number of transferable embryos per cycle. The lowest number of COC to result in a pregnancy in our series was six. More than 85% of the pregnancies occurred in cycles in which at least 9 COC were retrieved. The boundary point between good and bad prognosis was located at 9 COC. A significantly higher proportion of the cycles with ⩾9 COC compared with cycles with <9 COC led on to embryo biopsy and embryo transfer. The number of biopsied embryos per retrieved COC did not differ between cycles with <9 COC and cycles with ⩾9 COC. On the other hand, in the cycles with ⩾9 COC the number of biopsied embryos was 3-fold higher than in cycles with <9 COC. Although the number of transferred embryos per COC was similar in both groups, almost three times as many embryos per cycle were transferred in cycles with ⩾9 COC than in cycles with <9 COC. The difference in outcome between the bad responders and good responders can be explained in terms of mathematical differences in the absolute number of oocytes and embryos after each stage of the PGD procedure. Good responders do not score better than bad responders because of a better fertilization and biopsy rate after ICSI, but because of a notably higher absolute number of genetically healthy embryos, with a consequently higher number of embryos per transfer. We have demonstrated that pregnancy rates increase gradually with increasing numbers of embryos per transfer. The decrease of the total number of retrieved COC that reaches each stage of the PGD procedure (Figure 4) explains the association between the number of COC and outcome of the PGD cycle. Less than 20% of the retrieved COC resulted in an unaffected embryo. The cycle efficiency index (CEI), i.e. the proportion of retrieved oocytes to produce embryos which can be transferred or frozen, was only 16.2% (137 transferred embryos and 48 cryopreserved embryos out of 1140 COC). Meniru and Craft (1997) observed

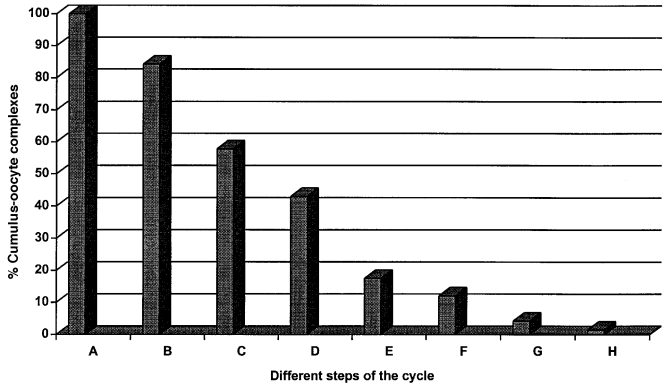

Figure 4. Number of oocytes and embryos in each step of the preimplantation genetic diagnosis cycle expressed as percentage of the number of retrieved cumulus–oocyte complexes (COC). A = retrieved number of COC; B = injected oocytes (4×IVF); C = 2 PN oocytes; D = biopsied embryos; E = genetically healthy embryos: F = transferred embryos; G = cryopreserved embryos; H = implanted embryos.

a mean CEI of 36% in assisted reproduction treatment cycles without PGD. Although the CEI between the groups with <9 and $\geqslant 9$ COC (13.7% and 16.5% respectively) did not differ significantly in our series, the substantial difference in the number of transferred embryos per cycle was the main reason why chances of becoming pregnant increased significantly when $\geqslant 9$ COC were retrieved. Moreover, when a higher number of unaffected embryos was obtained, we were able to select the embryos with the best morphology and those which continued to cleave after they had been biopsied. The transfer of a higher number of good-quality and further-cleaving embryos leads inevitably to higher rates of implantation and pregnancy.

It is appropriate to cancel the cycles of low responders. Although cost–benefit analysis is not the topic of this paper, we assume that by cancelling the cycles of low responders, pointless high costs associated with this time-consuming and labour-intensive technique can be avoided. Moreover, the stress may be lower in cycles in which many embryos are available. Cycles should be cancelled early in the follicular recruitment phase and not just before HCG administration. When the cycle is cancelled at the time that a low number (<9) of follicles start to grow, long stimulation periods—which are tiring and stressful for the patients and ultimately have poor success rates—can be avoided. Although the exact number of COC that will be retrieved during oocyte retrieval cannot always be predicted, markers such as serum oestradiol concentration and the number of recruited follicles on vaginal ultrasound offer an acceptable estimation. Female patients undergoing a PGD cycle should be stimulated according to an aggressive step-up protocol in order to recruit as many follicles as possible. If ovarian response is insufficient, a new cycle with an adapted dose of HMG or another drug should be initiated. Recombinant FSH, a very pure FSH preparation synthesized by Chinese hamster ovary (CHO) cell lines transfected with genes encoding human FSH (Howles, 1996; Olijve *et al.*, 1996) might be an alternative for these patients. Recombinant FSH reveals the same bioactivity and pharmacokinetics as natural FSH (Mannaerts *et al.*, 1993; de Leeuw *et al.*, 1996; Howles, 1996). It has been demonstrated in several multicentre studies that the use of recombinant FSH in different long protocols with GnRHa was associated with a higher number of retrieved COC and lower FSH requirements than were urinary FSH preparations (Out *et al.*, 1995; Recombinant Human FSH Study Group, 1995; Bergh *et al.*, 1997). Although this property of recombinant FSH might be a major advantage in PGD cycles, prospective trials comparing the efficacy of recombinant FSH and HMG are necessary to evaluate if more day 3-cleaved embryos are obtained with recombinant FSH than with HMG.

We demonstrated that the number of transferred and cryopreserved embryos per cycle was not affected by the origin of the tested genetic disorder. On the other hand, the distribution of unaffected and affected biopsied embryos was influenced by the origin of the disease. Surprisingly, this distribution did not follow Mendel's laws. Some 63% of the biopsied embryos in the group of monogenic recessive disorders were either homozygous normal or heterozygous normal. According to Mendel's laws, 75% of these embryos should be unaffected. The difference can be attributed to the fact that in some cycles for cystic fibrosis the two partners had different mutations. In these cycles, the embryos were tested for only one mutation, so that only 50% rather than 75% of these embryos were diagnosed as unaffected. In the group of monogenic dominant disorders, 31% of the biopsied embryos were unaffected instead of 50%. The semi-informativity of the maternal and paternal healthy gene in some Steinert cycles accounted for this difference. Similarly, in the group of sexing for X-linked disorders the proportion of healthy female embryos did not correspond to the expected 50%. The presence of binucleated embryos and embryos with a chromosome 18 aneuploidy, which was tested simultaneously, resulted in only 31% healthy female embryos. A sex-chromosome aneuploidy was inherited in 45% of the Klinefelter and 47,XXX embryos. The results per cycle did not differ significantly according to the tested disease. We believe that ovarian stimulation should not be individualized according to the inheritance pattern of the disease. High numbers of COC are required in every category of tested disorders.

Because the procedure requires a high number of oocytes, the patient will be put at risk of developing ovarian hyperstimulation syndrome (OHSS; Navot *et al.*, 1988). Cryopreservation of embryos so as to delay the transfer to a later date in patients with extremely high serum oestradiol concentrations and a high number of COC has been proposed in order to reduce the risk of OHSS (Navot *et al.*, 1993). Until now, very little information has been published on the in-vitro and in-vivo developmental effects of freezing and thawing of biopsied embryos in humans. In contrast, in a mouse model it has been ascertained that cryopreservation of embryos biopsied at the 4- to 8-cell stage has no detrimental effect on in-vitro blastocyst formation and in-vitro developmental potential when up to two blastomeres are removed (Wilton *et al.*, 1989; Krzyminska and O'Neill, 1991; Liu *et al.*, 1993). In our series, no pregnancies were achieved after frozen–thawed embryo transfer. Although the number of frozen–thawed PGD embryo transfers was far too low for us to draw any conclusion, we

fear that as well as the unknown cryo-effects, the implantation capacity of an embryo which is already jeopardized by the biopsy may be very low. For this reason, clinicians have to weigh carefully the risk of developing OHSS against the chances of a successful PGD treatment in a fresh cycle and cryo-cycle.

We demonstrated a clear positive correlation between the outcome of PGD cycles and ovarian responsiveness expressed as the number of retrieved COC. The impact of the number of COC can be explained in terms of the number of biopsied embryos and number of homozygous or heterozygous normal embryos. An ongoing pregnancy rate of 20.9% instead of 17% would have been achieved if we had cancelled all PGD cycles with <9 COC. In PGD patients, it is of utmost importance to monitor ovarian response accurately by means of pelvic ultrasound and serum oestradiol concentrations in order to retrieve at least 9 COC. On the other hand, two pregnancies ensued in two couples with respectively 6 and 8 COC. For this reason, we propose to cancel the cycles in which an egg retrieval of <6 COC may be expected. When 6–8 COC are expected, the option of cycle cancellation or cycle continuation should be discussed with the couple, taking into consideration the poor prognosis of cycles with <9 COC. After cancellation, an adapted stimulation protocol may increase the chances of conception in subsequent cycles. It is clear that bad responders do not benefit from PGD; thus, PND, gamete donation or adoption are the only acceptable alternatives to prevent the birth of an affected child in these couples.

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