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Double-Factor Preimplantation Genetic Diagnosis: Preliminary Results

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1. Introduction

Preimplantation Genetic Diagnosis (PGD) was first employed successfully for a monogenic disease detection almost 20 years ago (Handyside *et al.*, 1990). PGD was also applied to screen for chromosomal abnormalities in couples at risk of aneuploidy (i.e., Preimplantation Genetic Screening: PGS) six years later (Munne and Weier, 1996, Verlinsky *et al.*, 1996, Verlinsky *et al.*, 1996). Currently, both approaches have been extensively used worldwide with more than 2,000 scientific publications.

Briefly, PGS aims for the selection of euploid embryos to transfer, aiming to increase their implantation rate. FISH for 9 chromosomes is mostly the technique applied in PGS. It seems, however, that according to recent publications PGS may not be useful (Staessen *et al.*, 2004, Staessen *et al.*, 2008, Hardarson *et al.*, 2008, Mastenbroek *et al.*, 2007). In fact, analyzing the latest data presented by the European Society of Human Reproduction and Embryology (E.S.R.H.E.), on average just 27.4% of the transferred PGS-selected embryos implant (3,926 positive heartbeats / 14,325 transferred embryos)(Harper *et al.*, 2010). On the other hand, after 167,192 ART cycles in Europe using ICSI, the pregnancy rate is 29.8% (Andersen *et al.*, 2008). Obviously, PGS patients differ from ICSI patients since the first ones suffer from repetitive implantation failure (RIF) or have an advanced maternal age (AMA), but the low implantation rate obtained in these patients still remains as a problematic issue.

But, also referring to the E.S.H.R.E. data, the implantation rate in patients undergoing PGD for a monogenic disease, in which the maternal age is not at risk of producing aneuploid

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gametes, is 15.2% (1067 positive heartbeats /7,035 transferred embryos) (Harper *et al.*, 2010). It appears clear, then, that an increase of the implantation rate would not only be advantageous for the PGS patients but also for the PGD ones.

In PGS patients, in order to increase the implantation rate, comprehensive chromosomal analysis techniques have been proposed to completely karyotype the embryo to be transferred. One of these techniques is Comparative Genomic Hybridization (CGH), which has been applied widely in PGS (Keskintepe *et al.*, 2007, Sher *et al.*, 2007, Voullaire *et al.*, 2002, Wells *et al.*, 2002, Wilton, 2005), achieving up to a 74%-80% rate of implantation. Two different cells can be analyzed by CGH-PGS, the first polar body (1PB) and the blastomere (BL). Both options have their advantages and disadvantages; the use of 1PB-CGH permits the diagnosis of the cell within the same IVF cycle, as the CGH procedure requires up to 4 working days, but the result obtained only reflects indirect information about the oocyte, despite it being known that it is during the first meiotic division when most embryonic aneuploidies occur (Hassold and Hunt, 2001, Nicolaidis and Petersen, 1998). On the other hand, BL-CGH gives information about the embryo directly, but it cannot be analyzed during the same IVF cycle and, therefore, involves a cryopreservation step followed by a defrost step of euploid embryos prior to transfer.

Other techniques that allow for a full karyotype analysis and that are potentially useful to increase implantation in PGS patients are array-CGH or SNP-array, which permits a diagnosis within the IVF cycle and investigates the embryo directly, as it is applied to blastomeres. So far, several publications have tested the efficiency of array-CGH on single cells with promising results (Fiegler *et al.*, 2007, Hu *et al.*, 2004, Le Caignec *et al.*, 2006, Wells *et al.*, 2004, Handyside *et al.*, 2009). Recently, it has been applied clinically after blastomere or polar body biopsy, obtaining six pregnancies (Hellani *et al.*, 2008, Fishel *et al.*, 2009). Although array-CGH is a capable technique, more research must be done to validate it and monitor any increase in the implantation rate. Toward this aim, the ESHRE PGS Task Force have proposed a multicenter randomized control trial to assess the possible positive effect on implantation of the PGS on polar body using a 24-chromosome detecting technique as array-CGH (Geraedts *et al.*, 2009).

Now considering the enhancement of the implantation rate on patients undergoing PGD for a monogenic disease (PGD-patients), several approaches have been published with this aim. A methodology called Cell Recycling, i.e., FISH analysis of a blastomere and posterior PCR amplification of the very same cell, was described as a possible option for selected the embryos both genetically and cytogenetically (Thornhill *et al.*, 1994), but it was demonstrated that the protocol increased the allele drop-out (ADO) rate, thus reducing its diagnostic robustness (Rechitsky *et al.*, 1996).

Another approach is the Double-Factor PGD (DF-PGD) where the 1PB is analyzed using CGH and a single blastomere of the produced embryo is biopsied in order to diagnose the monogenic disease. This method permits the double selection of the embryos for being free of the family disease plus having originated from a potentially euploid oocyte. In addition, as 1PB-CGH is used, the diagnosis is performed within the same IVF cycle, avoiding the need of cryopreservation. The DF-PGD has been previously applied on two couples, carriers of cystic fibrosis and von Hippel-Lindau syndrome, respectively, achieving the birth of three healthy children (Obradors *et al.*, 2008, Obradors *et al.*, 2009).

The aims of this work are to assess the feasibility and application of the DF-PGD after two year of experience and ten clinical applications and, moreover, to determine if it is a valuable tool to increase the implantation rate in PGD for monogenic-disease patients.

2. Materials and methods

Patients

Over two years, a total of eight couples affected by a monogenic disease participated in this study after fully understanding the protocol and the signing of an approved-consent form. The results of two of them were previously published (Obradors et al., 2008 and 2009) (Table 1). Two of the couples repeated the DF-PGD clinical protocol after no pregnancy on the first attempt.

The mean age of the patients was 35.1 years old. For comparison proposes, the patients were classified into two groups depending on the maternal age: four patients were classified with advanced maternal age (AMA) (mean age of 39.2 y.o.) and the other 6 patients were ≤ 35 y.o. (mean age of 32.3 y.o.).

Most of the couples (6 out of 8) were unaffected carriers of Cystic Fibrosis (CF, OMIM #219700) with an affected child. Considering the other two couples, one was affected by von Hippel-Lindau syndrome (VHL, OMIM #193300), a dominantly-inherited family cancer syndrome, and the other couple was an unaffected carrier of Angelman Syndrome (AS; OMIM #105830).

A summary of the mutations affecting each couple can be also found in Table 1.

Monogenic disease detection protocol

In order to minimize misdiagnosis and increase protocol robustness, two independent diagnoses were performed. A direct diagnosis of the causative mutation and an indirect diagnosis using informative Small Tandem Repeats (STRs) were optimized for each couple. The causative mutation detection, mostly point-mutations, was achieved after two rounds of nested-PCR amplification of a region surrounding the mutation using specific primers designer using the Primer3 website (http://frodo.wi.mit.edu) (Table 2). After PCR amplification, the mutant site was interrogated using the MiniSequencing Reaction (Snapshot Multiplex Kit, Applied Biosystems; CA, USA). During the reaction, a mutation-specific primer anneals a base before the point-mutation site; then, a polymerase incorporates a single ddNTP fluorescently labeled (Fiorentino *et al.*, 2003). The product of the reaction (1µL) is analyzed in a DNA sequencer in order to detect the presence or absence of the causative mutation.

In order to detect mutations involving short deletions, such as ΔF508 mutation in the CFTR gene which is causative of Cystic Fibrosis (CF), where three nucleotides are deleted in the mutant allele, a pair of fluorescent primers was designed to anneal on both sides of the deletion in order to detect the fragment analysis variation, between the alleles. After PCR amplification of genomic DNA of a CF carrier and analysis on a DNA sequencer, two different peaks appear, a wild-type peak at 94 bp followed by the mutant peak at 91 bp.

As commented upon before, an indirect diagnosis using STRs was also applied. A minimum of four STRs were found surrounding each of the genes causatives of the diseases. The STRs were chosen using the NCBI database (http://www.ncbi.nlm.nih.gov/mapview) according to the following criteria: to contain a tetranucleotide repetition core, to be located as close as possible to the gene (upstream or downstream) and to have the highest heterozygosity value. Once they were chosen, the fluorescent dyes of the forward primers of each STR were selected in order to avoid overlapping of the expected allele size for all STRs of the same disease, so they could be amplified and simultaneously analyzed if required.

Couple	Maternal age	Disease	Gene	Mutation	Direct detection	Indirect detection
Obradors et al. 2009	30	Von Hippel-Lindau, Dominant	VHL	P: R161Q	MiniSequencing	D3S1675 D3S1537
A	32	Cystic Fibrosis, Recessive	CFTR	P: dF508 M: dF508	Fragment analysis	INTRAGENIC D7S1799 D7S1817 D7S3025 D7S2847
A'	32	Cystic Fibrosis, Recessive	CFTR	P: dF508 M: dF508	Fragment analysis	INTRAGENIC D7S1799 D7S1817 D7S3025 D7S2847
B	32	Cystic Fibrosis, Recessive	CFTR	P: dF508 M: dF508	Fragment analysis	INTRAGENIC
C	33	Cystic Fibrosis, Recessive	CFTR	P: 712-1GtoT M: dF508	MiniSequencing and Fragment analysis	D7S1799 D7S3025 D7S2847
Obradors et al. 2008	35	Cystic Fibrosis, Recessive	CFTR	P: 3408C>A M:3849+10Kb (CtoT)	MiniSequencing	INTRAGENIC D7S1799
D	37	Angelman Syndrome, Dominant	UBE3A	M: K109X	MiniSequencing	D15S817 D15S1513
E	38	Cystic Fibrosis, Recessive	CFTR	P:1811+1.6KbAto G M:2711delT	MiniSequencing	D7S1799 D7S1817
F	41	Cystic Fibrosis, Recessive	CFTR	P: dF508 M:dF508	Fragment analysis	INTRAGENIC D7S1799 D7S2847
F'	41	Cystic Fibrosis, Recessive	CFTR	P: dF508 M:dF508	Fragment analysis	INTRAGENIC D7S1799 D7S2847

Table 1. Diseases and causative mutations of the eight couples included in the present DF-PGP program. The mutation detection methods and the informative STRs to perform direct and indirect analysis, respectively, are also included. The dF508 mutation is detected by the 3-bp difference between the mutant and the wild-type alleles

To assess the diagnostic utility of the STRs, genomic DNA extracted from peripheral blood or from buccal cells was obtained from each member of the couple. The STRs were classified as being 100% informative if the both in the couple were both heterozygote, or to be partially informative if one of the couple was homozygote and the other one heterozygote for the same STR or, finally, not informative if both were homozygote. In order to detect which of the couple's alleles were linked to their mutated copy of the gene, DNA from an affected child or first-degree relatives was also analyzed.

With the aim of avoiding misdiagnosis due to the recombination process between the selected STRs and the gene of interest, a minimum of two 100% or partially informative

Primer	Forward (5'-3')	Reverse (5'-3')
K109X-outer	GCCTAGAATGTTTGGCTGTTT	CTCTTCCAATAACACGGATTAAA
K109X-inner	GAACTTTTTGCAACAGAGTAAACA	ATCCTCTCTTTCTCTACATAATTCAAG
K109X-miniseq	AGATGTGACTTACTTAACAGAAGAG	-
dF508	6FAM-TGGAGCCTTCAGAGGGTAAA	TGGGTAGTGTGAAGGGTTCAT
712-1 G to T-outer	TGTTAGTTTCTAGGGGTGGAAGA	AAAGGAGCGATCCACACG
712-1 G to T-inner	GACACCTGTTTTTGCTGTGC	<u>AAATGTGCCAATGCAAGTCC</u>
1811+1.6Kb-outer	AAAGTTTTGCCATTGGTTTTT	AAAGATGAAGACACAGTTCCCATA
1811+1.6Kb-inner	TTGTGTGCTGAATACAATTTTCTTT	AAAGATGAAGACACAGTTCCCATA
1811+1.6Kb-miniseq	AGAGAATCCTATGTACTTGAGAT	-
2711delT-outer	GGAGAGCATACCAGCAGTGA	AAGCACCAAATTAGCACAAAA
2711delT-inner	TTCGATATATTACTGTCCACAAGAGC	<u>AAGCACCAAATTAGCACAAAA</u>
D15S1513	6FAM-GAACTGGGGAAAATCAGGAT	ACACATTTTAAGCCAGCAGC
D15S817	6FAM-GCACCAATAGGCTAGACACG	AGCTTTACATGGCATGTGGT
D7S2847	HEX-TCACCTTCAGAAAGTATTGCC	TGAGGTGTTTCTCCAAGCTC
D7S3052	6FAM-AGTGAGACTAGCAGATGTACTTGG	GCCTCCCCATTTCATCTATT
INTRA	HEX-CAAGTCTTTCACTGATCTTC	TGAGCAGTTCTTAATAGATAA

Table 2. Sequences of the primers used in this study. The underlined primers were also used as MiniSequencing primers, as their 3' end hybridizes a base before the point-mutation. The other primers' sequences are cited in the previous applications of DF-PGD.

STRs located on each side of the gene (upstream and downstream) were selected for the PGD protocol, or in the case of intragenic STRs, a single STR was considered.

Once both the STRs and the mutation-specific primers were chosen, the PGD protocols were optimized using genomic DNA from one of the patients. Briefly, a multiplex PCR containing the fluorescent primers for the STRs amplification, plus the first-round unlabeled primers for mutation detection was applied in most of the cases. When optimizing the dF508 CF mutation, its primers were fluorescently labeled in order to detect the deletion, as described before. Between 0.5-1 µL of product of this PCR multiplex was used as a template for the second round of amplification of the mutation-specific primers. One microliter of the PCR amplification product was used in a DNA sequencer to assess STRs efficiency on the multiplex, whereas agarose gel analysis was enough to detect to correct amplification of the mutation-specific loci.

When the multiplex was efficient in genomic DNA, it was tested in whole genome-amplified (WGA) single cells, usually cultured fibroblast. The Multiple Displacement Amplification (MDA) (Genomiphi, HE Healthcare; Buckinghamshire, UK) was used following the manufacturer's protocol, but with a previous step of alkaline lysis of the single cell, as described before (Obradors et al., 2008 and 2009, Spits et al., 2006). Finally, and in order to describe the allele drop-out (ADO) rate, 30 patients' lymphocytes were amplified with the optimized protocol.

In order to minimize the presence of contamination due to exogenous DNA, all PCR mixes which included primers, Taq polymerase and buffers were performed on a sterile hood. While the target DNA, i.e., MDA products, PCR amplicons after a first-round amplification and DNA extracted from peripheral blood or from buccal cells were introduced into a separate sterile hood in another room.

A report, the informative study report, including the STRs analyzed, the mutation detection procedure and ADO rate described for each couple, was sent to the IVF centers, which scheduled a date for the PGD case according to the patient's stimulation cycle.

3. First polar body comparative genomic hybrydization

The 1PB-CGH procedure has been extensively described previously by our group (Obradors et al., 2008 and 2009). Briefly, the 1PB was washed four times with sterile PBS/0.1%PVP, in order to avoid potential contamination from cumulus cells, and it was then placed in a 0.2mL PCR tube. Cell lysis (1 µl of sodium dodecyl sulfate (17 µM) and 2 µl of proteinase K (125 µg/ml)) was required to facilitate the cell genomic-DNA liberation. Afterwards, the cellular DNA was amplified by means of DOP-PCR, a WGA technique described to produce a sufficient amount of DNA to perform a CGH (Telenius et al., 1992, Voullaire et al., 1999, Wells et al., 1999). Briefly, the DOP-PCR reaction tubes contained 1X Buffer, 2 µmol/l DOP primer (CCGACTCGAGNNNNNNATGTGG), 0.2 mmol/l dNTP and 2.5U of SuperTaq Plus polymerase (Ambion, USA) in a final volume of 50 µl. The tubes were placed in a thermocycler and underwent the following program: 94ºC for 4.5 min; eight cycles of 95ºC for 30s, 30ºC for 1.5 min and 72ºC for 3 min; 40 cycles of 95ºC for 30s, 56ºC for 1 min and 72ºC for 3 min with a final extension step of 72ºC for 8 min. After DOP-PCR amplification, fluorescent labeling with Spectrum-Red of the DOP-PCR product was performed by the Nick Translation Kit, following the manufacturer's indications (Vysis; Downers Grove, USA). With the purpose of obtaining reference DNA to compare with the 1PB (test DNA), between twelve to fifteen tubes containing three euploid female fibroblasts each were lysated and amplified like the 1PBs, but labeled with Spectrum-Green instead. The reference DNA produced from these twelve to fifteen cells was mixed in a single tube, kept at -20ºC and used as reference DNA when required. The resulting reference-DNA mix avoids intrinsic cell-dependent amplification differences that could bias the CGH results and provides an intense homogeneous fluorescent signal.

Both test and reference DNA co-precipitated with Cot-1-DNA, which blocks repetitive DNA sequences such as telomeres and centromeres. The resulting cytogenetic probe hybridized during 44 hours into a slide of euploid lymphocyte metaphases. After that, the slide was washed to remove unspecific hybridization and analyzed using an epifluorescence microscope. Ten lymphocyte metaphases were captured and karyotyped using the Metasystem's software to obtain a CGH profile. When the fluorescence ratio (test/reference) of a CGH profile for a specific chromosome, reported by the software, was < 0.8, the chromosome was lost in the DNA test, whereas when the ratio is > 1.2, a chromosome gain was present (Wells et al., 1999).

Potentially artifactually gained or lost chromosomes (i.e., Chromosomes 17, 19 and 22) were discarded from analysis when all three chromosomes were simultaneously gained or lost in the same cell; if not, they were considered as being real aneuploidies.

In the present work, no distinction between chromosome or chromatid gain or loss has been considered because, in our experience, after analyzing 1PBs and their corresponding MII

using CGH and FISH, respectively, the CGH-loss or CGH-gain profiles on the 1PBs were indistinguishably equivalent to losses or gains of either chromosome or chromatid in the MIIs (Gutiérrez-Mateo, et al., 2004).

4. Double-factor pgd clinical schedule

Exhaustive information about the methodology and schedule, based on the following timetable (Figure 1), of the Double-Factor PGD (DF-PGD) clinical application performed in the families included in this work has been previously published (Obradors et al., 2008 and 2009). On Day 0, retrieved oocytes were cleaned of cumulus cells to avoid cellular contamination during the PGD case. Following the IVF center's protocol, ICSI was performed on all MII oocytes, and immediately afterwards, the 1PB was biopsied using the partial zone dissection (PZD) procedure and washed four times with sterile PBS/0.1PVS as described above. In the cases, when the IVF center was located close to the lab (i.e., Barcelona), the cell lysis and DOP-PCR were done on Day 0; on the other hand, for cases located far from our lab (i.e., Madrid), the 1PB arrived in our lab in Bellaterra on Day +1, when the CGH protocol started. On the afternoon of Day +1, the CGH was placed to hybridize for 44 hours, thus, until the morning of Day +3. On Day +3, the CGH started to be analyzed; in general, a skilled technician took 1 hour to obtain a result for each CGH, so depending on the number of 1PBs it may last until Day +4. Also on Day +3, developing embryos reached the 6-8-cell stage and a single blastomere is biopsied using the same hole produced during the 1PB biopsy, but in order to increase the size of the hole, PZD or acid Tyrodes was used. Again, if the IVF center was located close to the lab (i.e., Barcelona), the PCR protocol started on Day +3, thus achieving the monogenic diagnosis results on Day +4. For IVF centers located in Madrid, the blastomere arrived on Day +4.

Late on Day +4, after the results from the DF-PGD were obtained, a written report was sent to the IVF center indicating not only the embryos free of each particular monogenic family disease, but also the results obtained after the complete cytogenetic analyses of the whole female chromosome complement. At that point, embryos free from the monogenic family pathology that were derived from oocytes that were predicted to be euploid and with a good morphological appearance can be selected and transferred. As is habitual in PGD, the family has been informed that performing a prenatal diagnosis in case of pregnancy would be recommended.

5. Results

First polar body and blastomere obtainment

After ovary stimulation and follicular puncture, 146 oocytes were obtained from the 10 DF-PGD clinical cases presented (mean of 14.6 per couple). One-hundred-thirty-two of them (90.4%) were at the MII stage, thus containing the 1PB, and were inseminated using the couple's spermatozoids by ICSI. Immediately there after, the 1PBs were biopsied, obtaining 112 1PBs; therefore, the 1PBs biopsy efficiency was 84.8% (112/132). The oocytes were incubated at 37ºC and checked for fecundation on Day +1. A total of 92 showed morphological signs of fecundation, obtaining a fecundation rate prior to the 1PB biopsy of 69.7%. As established in the IVF centers, embryos were checked for satisfactory division and quality on Day +2. On Day +3, 81 of the 92 (88%) fertilized oocytes did develop to 6-8-cell-stage embryos (mean of 8.1 embryos per cycle) and a single blastomere was biopsied.

Blastomere biopsy was achieved in all embryos (100%), hence 81 blastomeres were acquired. A summary of the cells obtained can be found in Tables 3 and 5.
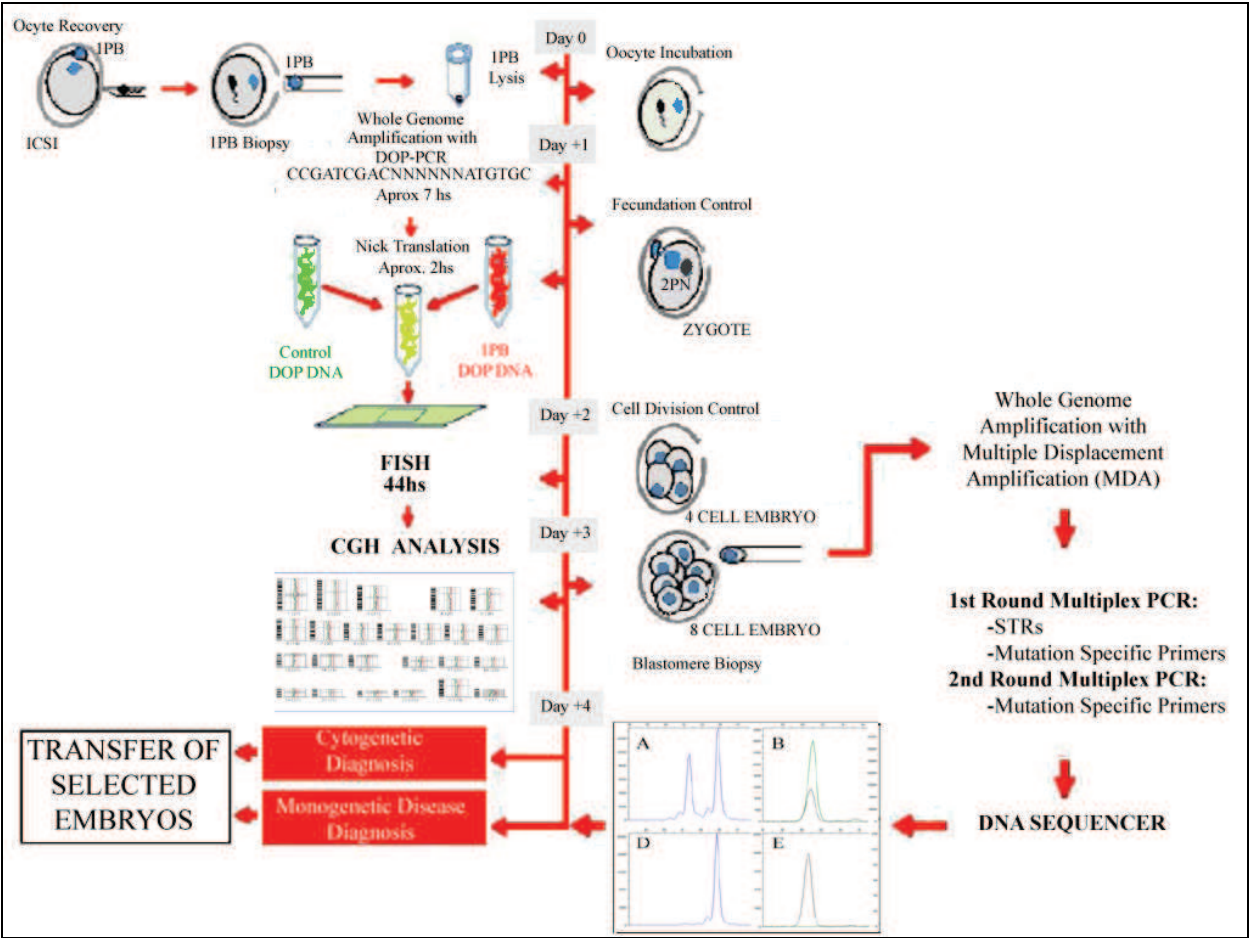

Fig. 1. Illustration showing the DF-PGD timing combined with the IVF procedure.

First polar body cgh results

Analyzable CGH profiles were obtained in 83 of the 112 1PB-CGHs performed, resulting in a CGH success of 74.1%. The remaining 29 1PB-CGHs, in which poor WGA amplification field were observed on the agarose gel, were not analyzable due to a faint hybridization was obtained. Probably it was due to the corresponding 1PB was fragmented or with bad morphology.

Thirty-five of the 1PBs were diagnosed as euploid, whereas the remaining 54.2% (45 out of 83) were aneuploid (Tables 3 and 4). No differences in the incidence of aneuploidy were observed between the two maternal age groups (56% in ≤ 35 years vs. 51.5% in > 35 years).

Aneuploidies involving from one to nine chromosomes were observed. All the 23 chromosomes were involved in aneuploidy. The chromosomes that most frequently were found to be aneuploid were Chromosomes 19 (eleven times), 18 and 16 (nine times), 1 (eight times) (Figure 2). No differences were observed concerning chromosome size, as from the 109 chromosomes involved in aneuploidy, 57 (52.3%) and 52 (47.7%) were classified into the A-C groups or into the D-G groups, respectively.

Only eight out the 45 aneuploid (17.8%) 1PBs would have been fully diagnosed using a FISH analysis for 9 chromosomes (13, 15, 16, 17, 18, 21, 22, X and Y), and a total of 20 (44.4%) of the aneuploid 1PBs would have been diagnosed incorrectly as being euploid (Figure 3). The remaining 17 1PBs (37.8%) would have been diagnosed as being aneuploid, but not all the chromosomal aneuploidies present would have been detected.

Couple	Maternal age	Oocytes retrieved	MII oocytes	1PB	Successful 1PB biopsy %	Analyzable 1PB-CGH	CGH Success %	EUPLOID 1PBs	ANEUPLOIDS 1PBs	ANEUPLOIDY RATE %
Obradors et al. 2009	30	12	12	10	100	12	100	8	1	11.1
A	32	16	16	13	81.25	13	92.3	3	9	75
A′	32	23	17	15	88.2	8	53.3	3	5	62.5
B	32	13	11	9	81.8	5	55.6	1	8	88.8
C	33	11	11	9	81.8	7	77.8	4	3	42.8
Obradors et al. 2008	35	6	5	5	100	5	100	3	2	60
D	37	7	5	5	100	3	100	1	2	66.7
E	38	33	32	23	71.9	13	56.5	4	9	69.2
F	41	11	11	11	100	11	100	7	4	80
F′	41	14	12	12	100	6	50	4	2	33.3
TOTAL	34.75	146	132	112	86.4	83	72.8%	38	45	54.2

Table 3. Summary of the oocytes retrieved from the eight couples included in the present DF-PGP program and cytogenetic results obtained after the 1PB-CGH analysis.

Monogenic disease detection results

All 81 blastomeres (a mean of 8.1 blastomeres per cycle) were amplified using MDA followed by a patient-specific mutation and STR detection multiplex-PCR as described. Six out of the 81 blastomeres (7.4%) did not provide a diagnostic result and thus were classified as non-diagnosed. Hence, as informative diagnostic result was notified in 92.6% of the blastomeres. Allele drop-out (ADO) affected 15.4% of the analyzed loci, and the PCR amplification efficiency was 93.3%.

Globally, of the 75 diagnosed blastomeres, 20 (26.1%) were homozygote wild-type, 21 (28%) homozygote mutant and the remanding 34 (45.3%) were diagnosed as heterozygote (Table 5). These ratios were not statistically different from the expected Mendelian inheritance rates.

Considering the studied monogenic diseases, 45 out of the 75 embryos were diagnosed as being non-affected (i.e., wild-type homozygotes in VHL and AS diseases, and both wild-type homozygotes and heterozygotes in CF).
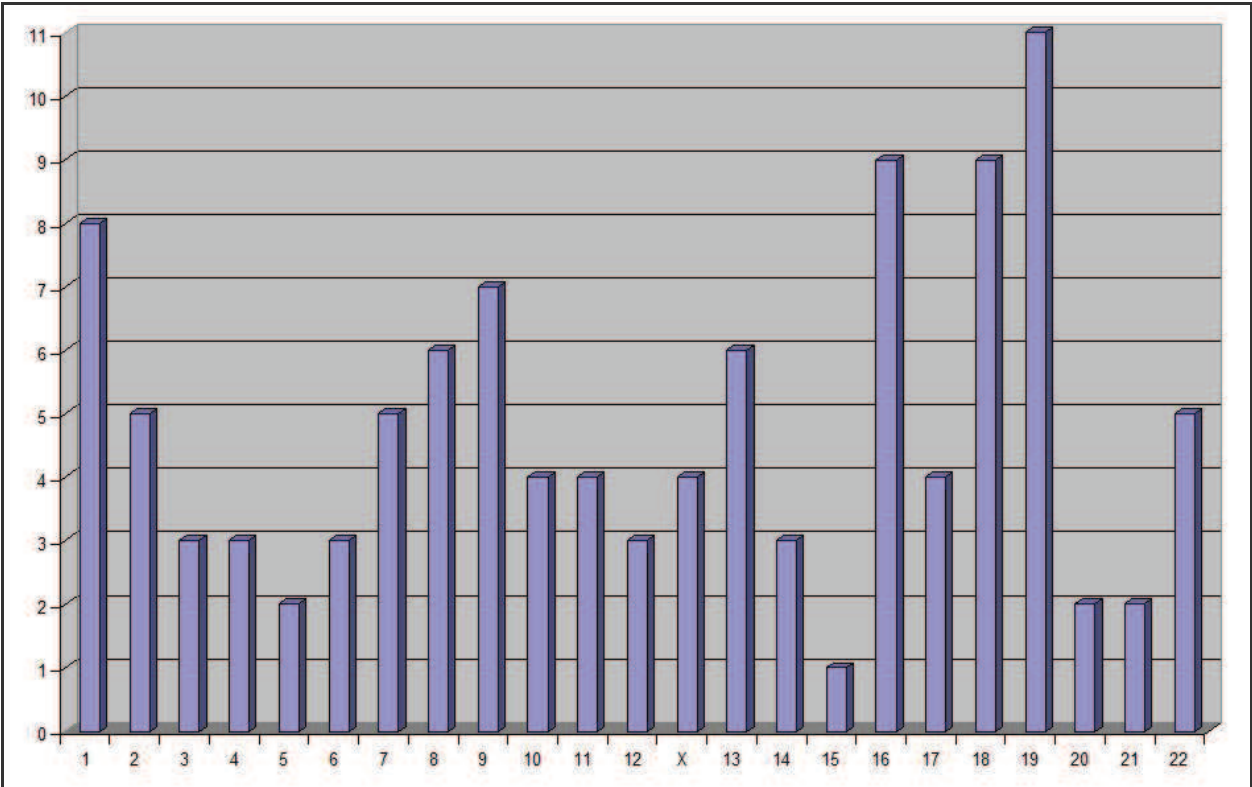
Fig. 2. Incidence of aneuploid chromosomes found in the 1PB-CGH results of the couples included in the present DF-PGP program.

Double-factor dgp outcome and implantation rate versus pgd

Of the 45 embryos free of the disease, in 35 the 1PB-CGH result was available, with the result that 20 of them (57.1%) originated from an aneuploid oocyte and the rest, 15 (42.9%), were potentially euploid. These 15 embryos, free of the monogenic disease and potentially euploid, were tagged as DF-PGD-transferable embryos. No informative 1PB-CGH profiles were obtained from the remaining 10 embryos free of the disease, so only the monogenic-disease diagnosis was performed; they were classified as conventional PGD-transferable embryos.

Consequently, 56 embryos were DF-PGD non-transferable, due to being undiagnosed or affected by the corresponding monogenic disease (6 embryos and 30 embryos, respectively), or due to having originated from aneuploid 1PBs (20 embryos) (Table 6).

On Day +5, according to embryo-quality criteria, nine out of the 15 and eight out of the nine DF-PGD-transferable and conventional PGD-transferable embryos were transferred to nine patients, respectively. Four of them received only DF-PGD-transferable embryos, two received only conventional PGD-transferable embryos and the remaining three patients received both types (Table 6).

Four pregnancies were confirmed with both the hCG test and positive fetal heartbeat. None of the pregnancies involved patients receiving simultaneously both types of embryos (DF-PGD-transferable embryos and conventional PGD-transferable embryos). A pregnancy which turned into the birth of a healthy girl was achieved in one of the patients with just PGD-transferable embryos transferred, and two pregnancies were achieved from the patients receiving only DF-PGD-transferable embryos, which resulted in the birth of three healthy children (one singleton and twins).
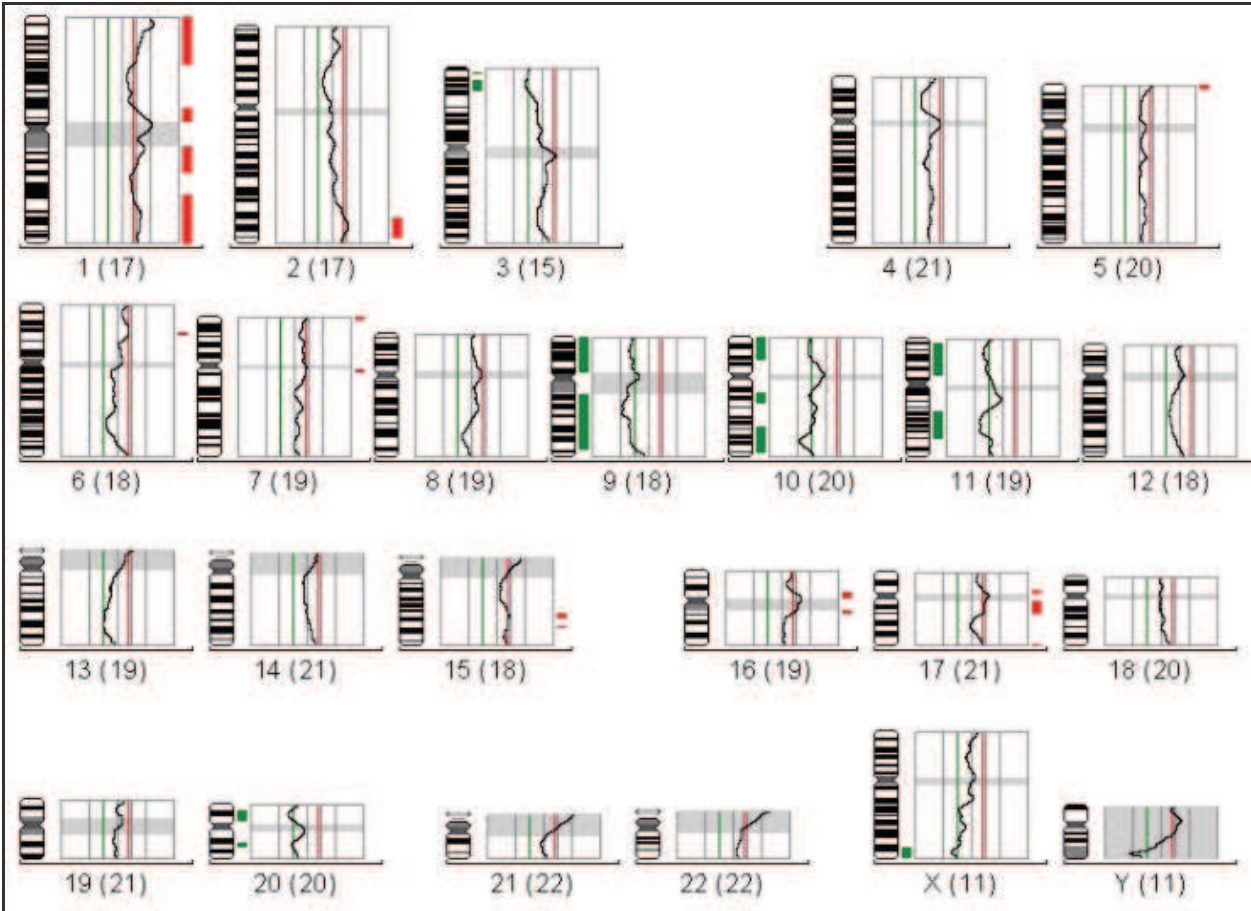
Fig. 3. Comparative Genomic Hybridization profile of 1PB#1 from Patient D, indicating a gain of Chromosome 1 and a loss of Chromosomes 9, 10 and 11. The 1PB would have been misdiagnoses as euploid if instead of CGH; a nine-chromosome FISH would have been used.

Due to most of the embryos were either at the morula stage or degenerated on Day +6, it was totally impossible to isolate blastomeres from the rejected embryos in order to perform a confirmation of both diagnoses.

Prenatal diagnosis has been performed on two out of three pregnancies, according to the parents' decision. In Couple C, an embryo diagnosed as a healthy homozygote turned out to be carrier of one of the copies of the mutation due to ADO of the mutant allele; consequently, the girl delivered was a healthy heterozygote, but a carrier of CF (Figure 4).

Taking into consideration the implantation rate for the two types of embryos, one out of eight embryos healthy for the monogenic disease but undiagnosed for aneuploidies (i.e., PGD-transferable embryos) did implant, achieving an implantation rate of 12.5%, whereas three out of nine healthy embryos diagnosed as potentially euploid (DF-PGD transferable embryos) did implant, meaning an implantation rate of 33.3%. The differences between the groups are not significant for Fisher's test (p= 0.576).

Considering maternal age, none of the six embryos transferred into patients with AMA (mean age of 39.2 y.o.) did implant, whereas four out of the eleven embryos transferred into the patient without AMA (mean age of 32.3 y.o.) implanted. These differences are found to be significantly different (p= 0.03).
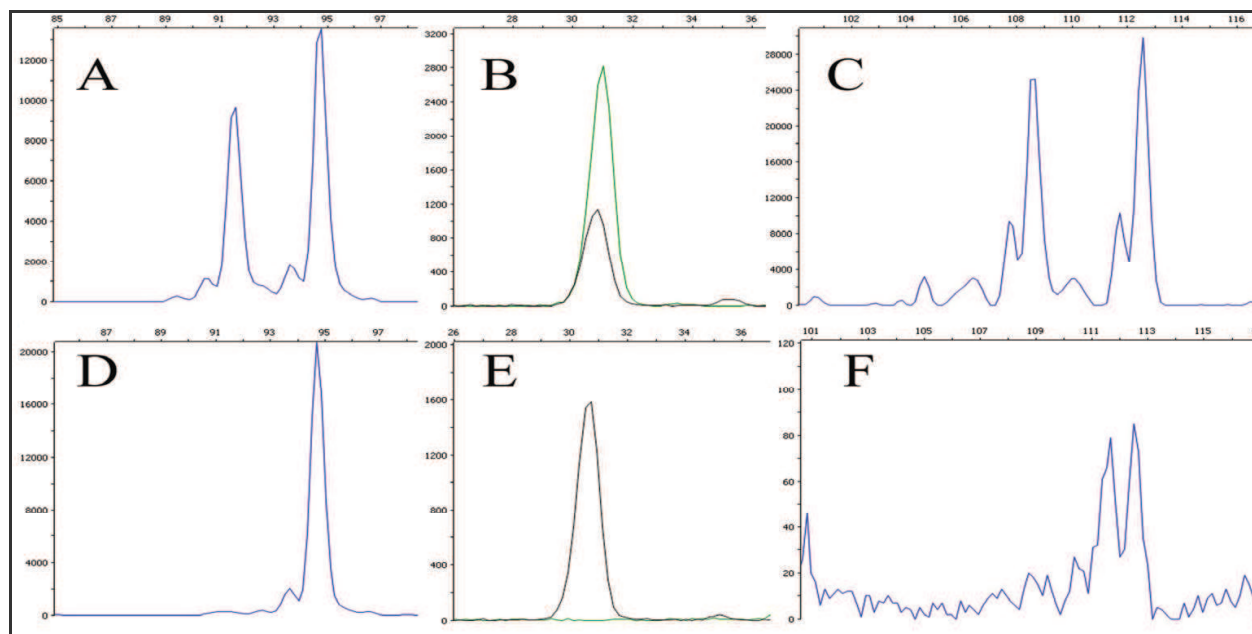
Fig. 4. Monogenic diagnosis results of embryos #4 and #5 of Patient C, carrier of CF. Embryo #4 was affected of both mutation, dF508 (A) and 712GtoC (B), and confirmed with the STR D7S3025 (C), whereas Embryo #5 (D, E, F) was free of both mutations. Embryo #5 was transferred to the patient, achieving a pregnancy. Prenatal diagnosis showed that the foetus carried the dF508 mutation. Therefore, two ADO events occurred in that blastomere, one in the dF508 locus and another in the STR D7S3025 (D and F).

6. Discussion

The embryo implantation rate is one of the most important values in IVF as long as the take-home-baby rate as the major aim of the clinicians is to achieve pregnancy in their IVF patients. Preimplantation Genetic Screening (PGS) appeared to satisfy this demand. It is well documented that aneuploidy affects between 50%-65% of first-trimester abortions (Hassold and Jacobs, 1984, Menasha *et al.*, 2005). Therefore, a positive selection of euploid embryos to transfer should be a useful tool towards an increase of their implantation rate. However, it has been proved that, apart of embryonic mosaicism, the screening of embryos for a limited number of chromosomes is insufficient Steassen et al., 2004 and 2008; Hardarson et al., 2008; Mastenbroek et al., 2007) and that a complete karyotype analysis may be needed to obtain satisfactory results (Sher et al., 2007).

Comparative Genomic Hybridization (CGH) and array-CGH are the main techniques used for comprehensive aneuploidy screening of embryos. The array-CGH has been tested lately for single cells and even applied clinically on blastomeres and on polar bodies, obtaining six pregnancies in nine couples with a clinical history of up to thirteen previous IVF failures (Hellani et al., 2009). One of the main advantages of array-CGH versus conventional CGH is that it is less labour-intensive, more informative and plus it allows embryo diagnosis on Day 4. On the other hand, its main drawback is the cost of the array-CGHs and the consequent processing and analysis equipment required. Thus, in anticipation of more studies of both economic and technical viability of the array-CGH, conventional CGH has been continuously applied with the objective of enhancing the implantation rate with implantation rate up to 68.9% Keskintepe, et al., 2002).

Aiming to contribute on the increase of the implantation rate, even in patients without a previous clinical history of IVF failure or AMA, our group introduced the Double-Factor PGD (DF-PGD), which allows for the selection of potentially euploid embryos with 1PB-CGH and moreover free of the corresponding family monogenic Obradors et al., 2008 and 2009). In the present manuscript we are including results obtained after two years of the application of DF-PGD.

Considering the DF-PGD protocol referred to here, one single blastomere was biopsied from all 81 6-8-cell embryos on Day 3, as recommended by the ESHRE PGD consortium in order not to compromise embryo viability (Thornhill *et al.*, 2005). All 81 blastomeres (a mean of 8.1 blastomeres per cycle) were amplified using MDA prior to PCR amplification with an efficiency rate of 92.59%, similar to the large series of MDA applications previously published, which analyzed 88 and 49 single cells, respectively (Burlet *et al.*, 2006, Renwick *et al.*, 2006). One of the main concerns about MDA is its high ADO rate, which, according to the same cited studies, affects between 25%-27% of the heterozygote cells. In this work, and also analyzing a comparable number of cells, the ADO rate obtained was appreciably low (15.4%). Despite that, on one of the patients (Couple E), the ADO rate found after analyzing their embryos was considerably higher (50%), most probably due to intrinsical problems with this case in particular, as blastomeres quality or transportation issues. If the data from this couple is not included for the ADO rate calculation, its value becomes 11.5%, similar to the obtained from unamplified single cells, and also close to the recommended for the ESHRE PGD consortium Thornhill et al., 2005). The differences in the ADO rates showed in this results compared with the previously published using MDA could be explained by the differences in the size of the PCR amplicons and the type or quality of the amplified cells (Wells, 2004). Unfortunately, and despite the low ADO rate, a misdiagnosis occurred affecting a healthy cystic fibrosis (CF) carrier who was wrongly diagnosed as being a wild-type homozygote.

In that particular case, the family carried two different CF mutations (ΔF508 and 712-1G to T); moreover, they were fully informative for one STR and semi-informative for two more STRs (only informative for one of the progenitors). After MDA and PCR amplification, the analysis showed wild-type alleles for both mutations, failure of amplification on the full informative STR and consistent results on the other two STRs, thus the embryo was diagnosed as being free of both CF mutations. Pregnancy was achieved in this couple, and following the group's recommendation, prenatal diagnosis was performed showing a foetus carrier of the ΔF508 mutation. Hence, two ADO events did occur in that cell, one affecting the ΔF508 mutation site and the other on the semi-informative STR linked to the ΔF508 mutation. This result may indicate that the blastomere analyzed probably was insufficiently lysed. Nevertheless, prenatal diagnosis is mandatory in all PGD cases in order to avoid improbable, but possible misdiagnosis.

In a recent publication, the ESHRE PGS Task Force has aimed for a proof of principle study by a multicentre RCT of the positive effect of implantation of the 1PB and 2PB analysis in the same IVF cycle using a whole chromosome analysis technique as CGH-array (Geraedts et al., 2009) in order to overpass the mosaicism issue and detected all the possible aneuploidies without the requirement of embryo cryopreservation.

In the present study, we have applied a similar approach to the PGS Task Force, also using polar body analysis with a fully comprehensive technique (1PB-CGH). In this case we have decided not the analyze the 2PB in order to avoid an additional second manipulation of the oocytes, after the 1PB biopsy has been performed and due to the first polar body's chromosomal material losing its quality progressively through time (Durban *et al.*, 1998). We

also considered inappropriate to wait, at least two hours, for the 2PB extrusion and to perform the biopsy of both polar bodies at the same time. It is obvious that exclusively analyzing the 1PB, meiotic errors produced during second meiotic division remain undetected (Kuliev *et al.*, 2005). Considering that the incidence of the female origin of aneuploidies has been widely described mainly due to errors in the first meiotic division (Hassold and Hunt, 2001, Nicolaidis and Petersen, 1998), we decided that our DF-PGD approach was more than appropriate neither the oocyte nor the embryo was compromised.

A total of 115 1PBs were biopsied from their corresponding oocytes on Day 0, but only 77 of them were analyzed by CGH since the producing embryo was diagnosed as being affected by the monogenic disease. Surprisingly, 54.2% of the 1PBs were aneuploid. This high rate is similar to that previously described in CGH studies of 1PBs-oocytes doublets of IVF patients Gutiérrez-Mateo et al., 2004 a and b). It is noteworthy that the patients that underwent this study were selected for monogenic disease detection, not for sterility problems, so the fact that their aneuploidy rate is similar to that of IVF patients emphasizes the importance of aneuploidy screening even in patients without fertility problems. Moreover, after dividing the patients into two age groups, ≤35 or >35 years old, a similar rate of aneuploidy was found between them (56% vs. 51.5%, respectively). These results, although higher, are comparable to the ones obtained on a previous study of our group after analysis, with CGH, 1PB-MII doublets from 53 IVF donors with a mean age of 26.1 years old (Obradors *et al.*, 2010). The study shows that, despite being produced by young women, 32.1% of the analyzed oocytes were aneuploid. Moreover, it demonstrates that almost 40% of the donors had at least one aneuploid oocyte.

This high aneuploidy rate found in young fertile women, concordant with that described in this manuscript, may be explained due to the ovarian stimulation process (Weghofer *et al.*, 2008, Weghofer *et al.*, 2008). This suggests that not only aged patients may benefit from aneuploidy screening with CGH, but also younger women without sterility problems.

Almost half of the detected aneuploid 1PBs (44.4%), some of them containing up to 4 chromosome abnormalities (such as 1PB#1 from Patient D), would have been diagnosed as being completely euploid if a nine-chromosome (13, 15, 16, 17, 18, 21, 22, X and Y) FISH had been used instead of CGH. This means that even performing an exhaustive FISH aneuploidy screening, almost half of the aneuploid embryos could still have been transferred into the patient's uterus, despite their intrinsic impossibilities to develop a pregnancy.

Consequently, in order to increase the implantation rate, not only a partial chromosome screening must be performed but also it must be done using a full-chromosome analysis technique such as CGH.

Referring to the data presented here, from the 45 developing embryos healthy for their specific monogenic disease, 20 of them originated from a potentially aneuploid oocyte diagnosed with 1PB-CGH, whereas 15 of them were diagnosed as being potentially euploid (tagged as DF-PGD transferable embryos). In the remaining ten embryos, no CGH results had been obtained, and they were classified as PGD transferable embryos.

Nine out of fifteen and eight out of ten embryos were transferred to the patients from the DF-PGD-transferable and PGD-transferable groups, respectively. Three patients (A', B, and E) received both types of embryos. Three from the nine and one out of the eight transferred embryos from each group implanted and developed into the birth of four healthy children. In none of the patients receiving both types of embryos did implantation occur. Also, none of the patients with AMA (mean age of 39.2 y.o.) became pregnant although six embryos were transferred; on the other hand, the four pregnancies achieved

did occur in younger patients after transferring 11 embryos. These differences are found to be significantly different (p= 0.03), and may be explained by other factors, rather than aneuploidy, that might affect the implantation rate in aged women like gynecological or immunological aspects, although more studies are required to reach clear conclusions.

Therefore, the implantation rate was 33.3% in the embryos doubly selected (DF-PGD transferable embryos) and 12.5% in the embryos selected as being of the monogenic disease but not screened for aneuploidy, a value that is very similar to the average value (15.2%) obtained in the last ESHRE Consortium Steering Committee recompilation (Harper et al., 2010). Despite the differences between the implantation rates of the DF-DGP transferred embryos being two times higher that the DGP transferred embryos are promising, these differences were not significant. Most probably, the reduced number of clinical applications presented in this work is not enough to have major differences.

In conclusion, although the DF-PGD-selected embryos did not obtain a significant increase of implantation compared with embryos undiagnosed for aneuploidy, it seems clear that comprehensive aneuploidy screening of oocytes using a PGD protocol might benefit the clinical outcome of their corresponding embryo. Therefore, in order to increase the implantation of embryos selected that are free of a monogenic disease, and until the routine assessment of CGH-array methodology, 1PB-CGH is the preferred technique to positively doubly select potentially euploid embryos.

7. Acknowledgements

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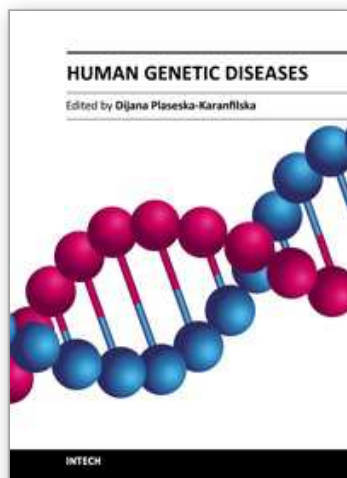
Human Genetic Diseases

Edited by Dr. Dijana Plaseska-Karanfilska

The genetics science is less than 150 years old, but its accomplishments have been astonishing. Genetics has become an indispensable component of almost all research in modern biology and medicine. Human genetic variation is associated with many, if not all, human diseases and disabilities. Nowadays, studies investigating any biological process, from the molecular level to the population level, use the "genetic approach" to gain understanding of that process. This book contains many diverse chapters, dealing with human genetic diseases, methods to diagnose them, novel approaches to treat them and molecular approaches and concepts to understand them. Although this book does not give a comprehensive overview of human genetic diseases, I believe that the sixteen book chapters will be a valuable resource for researchers and students in different life and medical sciences.

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