

## Article

# Maternal age, morphology, development and chromosome abnormalities in over 6000 cleavage-stage embryos



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Santiago Munné is the founder and co-director with Jacques Cohen of Reprogenetics. His group there focuses on identifying genetically normal embryos. Originally from Barcelona, Spain, Dr Munné gained his PhD in genetics from the University of Pittsburgh and joined Dr Jacques Cohen at Cornell University Medical College, New York in 1991. There he developed the first PGD test to detect embryonic numerical chromosome abnormalities. His work has been recognized by several prizes: in 1994, 1995 and 1998 from the Society for Assisted Reproductive Technology, and in 1996 from the American Society for Reproductive Medicine. Recently the PGD team has shown higher pregnancy rates in women of advanced age undergoing PGD. This team has performed more than 850 PGD cycles for translocations and over 8500 PGD cycles for chromosome abnormalities related to advanced maternal age. Dr Munné has more than 100 publications to his name, and is a frequent lecturer, both nationally and internationally, on his team's work and the field of preimplantation genetics.

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## Abstract

Previous studies assessing the relationship between embryo development, maternal age and chromosome abnormalities were either small or analysed mostly embryos not suitable for replacement. The present study includes >6000 embryos, including many suitable for replacement. Embryos with the best morphology and development were 44% euploid in patients younger than 35, decreasing to 21% in patients 41 and older. The worst morphology group had only 30% normal embryos from patients younger than 35, and 12% in embryos from patients 41 and older. Thus morphological analysis was able to improve the population of normal embryos only from 30 to 44% in the best of cases. Regarding specific abnormalities, 20% of embryos were aneuploid, 32% aneuploid plus other abnormalities, and the rest had post-meiotic abnormalities. Of those, only aneuploidy increased with maternal age. There were no big differences in the frequency of chromosome abnormalities depending on patient indication, within a similar age group. In summary, previous trends detected in suboptimal embryos were also confirmed in the best embryos for replacement. Although dysmorphism and advanced maternal age are both related to chromosome abnormalities, these parameters can yield at most <50% euploid embryos, and other techniques such as preimplantation diagnosis are required to ensure that only euploid embryos are replaced.

**Keywords:** advanced maternal age, preimplantation genetic diagnosis, recurrent pregnancy loss

## Introduction

The relationship between chromosome abnormalities, embryo morphology, and maternal age has been studied extensively (Munné *et al.*, 1995, 2004a; Magli *et al.*, 2001; Márquez *et al.*, 2000; Bielanska *et al.*, 2002a,b; Gianaroli *et al.*, 2007; Munné, 2006). These studies found that aneuploidy increases in cleavage stage embryos with maternal age, but there is no clear relationship between embryo development, dysmorphism and aneuploidy (Munné *et al.*, 1995; Márquez *et al.*, 2000). Conversely, post-meiotic abnormalities such as mosaicism, polyploidy and

haploidy increase with decreasing embryo development and with increasing dysmorphism (multinucleation, fragmentation, unevenness), but not with maternal age (Munné *et al.*, 1995, 2006; Márquez *et al.*, 2000; Munné, 2006; Magli *et al.*, 2007), with the exception of aneuploid embryos that are also mosaics (Munné *et al.*, 2002).

Although these trends are well documented, the data were mostly collected from embryos not suitable for replacement

because of morphological or developmental abnormalities, and only a small fraction of embryos studied were of a quality compatible with normal embryo transfer strategies. Thus, from the point of view of an embryologist trying to determine which embryo to replace, the most important information was missing, being the wider chromosome constitution of normal embryos. Since those studies, preimplantation genetic diagnosis (PGD) technology has evolved, with the current standard being the analysis of at least eight chromosome pairs.

The present study attempted to overcome the shortcomings of previous studies by including only embryos analysed for PGD, including a majority of embryos deemed replaceable or freezable by morphological and developmental standards and the best for replacement. In addition, all embryos included in this study had eight chromosomes analysed.

## Materials and methods

### Embryo source and classification

Embryos included in this study were from patients undergoing IVF treatment for infertility and PGD at the Institute for Reproductive Medicine and Science of Saint Barnabas Medical Centre between October 1997 and July 2006. The genetic analysis was performed by the same genetics laboratory, but this laboratory became a separate company (Reprogenetics) in 2002. For practical purposes concerning this study, the genetics laboratory was the same facility. The study was in accordance with guidelines set by the internal review board of the centre. Only cycles that had PGD for infertility were included, and none of the patients had a known previous history of translocations, or gene defects.

Embryos included in the study were all of monospermic origin developing from bipronucleate zygotes and were classified according to morphology and developmental rates into four groups: group 1 were embryos that on day 3 had not cleaved during a 24 h period and were considered arrested, regardless of morphology. These embryos nevertheless had 4 cells on day 3 and were considered suitable for biopsy and potential replacement if nothing else was available; group 2 were embryos that on day 3 had <6 cells but had cleaved in the previous 24 h. These embryos could have any morphology; group 3 included embryos that on day 3 had 6 or more cells, which had cleaved in the previous 24 h, but with >20% fragmentation, and/or with multinucleated blastomeres; and group 4 included embryos that on day 3 had 6 or more cells, with cleavage in the previous 24 h, <20% fragmentation, and no multinucleated blastomeres.

### PGD and re-analysis

Day 3 embryos were biopsied by zona drilling using acidified Tyrode's solution, and the embryos returned to culture as described elsewhere (Munné *et al.*, 2003). All of the embryos were at the 4- to 12-cell stage of development at the time of biopsy. All blastomeres were fixed individually following the protocol to minimize signal overlap (Munné *et al.*, 1996) and loss of micronuclei (Munné *et al.*, 1998a; Velilla *et al.*, 2002).

Fixed cells were sent to the genetics laboratory for genetic analysis. This was performed by fluorescence in-situ hybridization (FISH)

consisting of two consecutive hybridizations following previously published protocols (Munné *et al.*, 1998b). The first hybridization was performed with a cocktail of probes for chromosomes 13, 16, 18, 21 and 22, which is commercially available (Multivision PB; Vysis, UK). The second hybridization used an in-house mix of probes for chromosomes X, Y, 15 and 17 prepared as described previously (Munné *et al.*, 1998a).

If the specific signals for a chromosome were not clearly separated because they were too close to each other, a third hybridization using a telomeric probe for that chromosome was used, as described by Colls *et al.* (2004, 2006), in order to determine if the close signals represented one or two chromosomes. These analyses were performed from 2004 onwards.

When patient permission was granted, embryos found to be abnormal had all or most of their cells re-analysed. This was the only inclusion criterion applied. All embryos re-analysed were disaggregated on day 3 or 4 of development (as described in Márquez *et al.*, 2000), and all of their blastomeres were individually fixed on glass slides and genetically analysed using the same FISH methods described above. The re-analysis data were used to determine the frequency of false positives (embryos classified as abnormal that were in fact normal; **Table 1**), and to assess specific chromosome abnormalities more accurately.

Normal embryos that were in excess of the number considered suitable for replacement were usually cultured onward to day 5, when they were frozen if not arrested. Those not suitable for freezing could be re-analysed, but their results were considered biased since they were cultured to day 5, and if arrested they could have a higher chance of becoming polyploid and chaotic (Munné *et al.*, 1994). Thus, these embryos were not used to assess false negative rates.

**Table 1.** False positive error rate.

By re-analysis	By PGD
<i>Without third hybridization<sup>a</sup> (before 2004)</i>	
Normal	82
Abnormal	617
Total	699
% false positives	12
<i>With third hybridization<sup>a</sup> (2004 and after)</i>	
Normal	15
Abnormal	418
Total	433
% false positives	3
<i>Total</i>	
Normal	97
Abnormal	1035
Total	1132
% false positives	9

PGD = preimplantation genetic diagnosis.

<sup>a</sup>A third hybridization with probes for doubtful results was used from 2004 to date, helping to minimize errors (Colls *et al.*, 2004, 2006).

The analysis of a single cell allows overt classification of an embryo as normal or abnormal. This does not reliably differentiate between aneuploidy or mosaic embryos (most cells abnormal but each cell with a different chromosome constitution). Embryos were classified only as 'normal' (all informative chromosomes analysed with two signals) or 'abnormal' (with one or more informative chromosomes analysed with one, three or another number other than two signals).

After re-analysis, abnormal embryos could be reclassified chromosomally. The criteria to classify embryos as normal, aneuploid, extensive mosaic, polyploid or haploid based on FISH results of most or all the cells of an embryo has been previously described, and was followed here without modification (Munné *et al.*, 1998c). Mosaic embryos were classified into different mosaic subgroups following previous criteria (Munné *et al.*, 2002). The cut-off to classify an embryo as extensive mosaic was when it had  $\geq 3/8$  abnormal cells or 37.5%. Thus, for example, an embryo with six cells, three being abnormal and three normal, would be classified as extensive mosaic, but if it had two abnormal and four normal cells, it would be considered 'normal'.

## Statistical analysis

Since the variables of interest in this study were, for the most part, proportions of embryos possessing certain properties, the technique of logistic regression was used to analyse the data. This technique is simply one component of a family of analyses called generalized linear modelling (GLM). This particular analysis is fairly similar in principle to the more familiar analysis of variance, except for a more flexible use of distributional assumptions for the error, a prudent and appropriate use of variable transformations, and the use of maximum likelihood for the fitting process. Although the analyses were carried out on the logistic scale for purposes of presentation, all the summaries will be presented on the scale of proportions. The statistical package GENSTAT (version 2005) was used for the numerical calculations.

The following procedure was adopted in order to generate meaningful results relating to the effect of age on individual genetic abnormalities as presented in the results section (**Table 3**, see below), since only a sample of the previously diagnosed abnormal embryos were re-analysed.

**Table 2.** Chromosome abnormalities in embryos according to morphology and maternal age.

Group	Age (years)	Normal <sup>a</sup>	Abnormal <sup>a</sup>	Total	% normal
1	<35	15	35	50	30
	35–37.9	11	34	45	24
	38–40.9	21	64	85	25
	41 and up	8	58	66	12
	Total	55	191	246	22
2	<35	112	191	303	37
	35–37.9	91	247	338	27
	38–40.9	131	373	504	26
	41 and up	72	299	371	19
	Total	406	1110	1516	27
3	<35	40	90	130	31
	35–37.9	49	103	152	32
	38–40.9	66	159	225	29
	41 and up	35	179	214	16
	Total	190	531	721	26
4	<35	307	397	704	44
	35–37.9	337	464	801	42
	38–40.9	307	728	1035	30
	41 and up	219	812	1031	21
	Total	1170	2401	3571	33
Total by age	<35	474	713	1187	40
	35–37.9	488	848	1336	37
	38–40.9	525	1324	1849	28
	41 and up	334	1348	1682	20
	Total	1821	4233	6054	30

<sup>a</sup>For chromosomes X, Y, 13, 15, 16, 17, 18, 21 and 22.

Overall difference between age groups:  $P < 0.001$ ; differences by morphology/development:  $P < 0.001$  for group 4 versus groups 1, 2, and 3.

**Table 3.** Types of chromosome abnormalities analysed according to age group. The 97 embryos classified as errors were not included in this table.

Abnormality	Age group (years)				Total
	<35	35–37.9	38–40.9	>40	
Aneuploid (%)	27 (9.5)	28 (10.1)	78 (16.0)	70 (16.3) <sup>b</sup>	203
Aneuploid + haploid	0	1	0	0	1
Aneuploid + mosaic (%)	40 (14.0)	43 (15.5)	110 (22.5)	123 (28.7) <sup>c</sup>	316
Aneuploid + polyploid	0	3	3	6	12
Total aneuploidy (%)	67 (23.5)	75 (27.0)	191 (39.1)	199 (46.4) <sup>c</sup>	
Haploid	6	4	24	5	39
Extensive mosaic (%)	81 (28.4)	68 (24.5)	118 (24.2)	122 (28.4)	389
Polyploid (%)	15 (5.3)	12 (4.3)	27 (5.5)	21 (4.9)	75
Total post-meiotic <sup>a</sup> without aneuploidy (%)	102 (35.8)	84 (30.2)	169 (34.6)	148 (34.5)	
Total post-meiotic <sup>a</sup> (%)	142 (49.8)	131 (47.1)	282 (57.8)	277 (64.6) <sup>c</sup>	
Total abnormal	169	159	360	347	1035
Total normal <sup>d</sup>	116	119	128	82	
'Notional' total embryos <sup>d</sup>	285	278	488	429	

<sup>a</sup>Post-meiotic abnormalities: extensive mosaicism, haploidy, polyploidy.

<sup>b</sup>Denotes significant difference,  $P < 0.01$  and  $P < 0.001$  respectively.

<sup>d</sup>Theoretical estimates computed as described in the text.

The sampling fraction for re-analysis was 0.2445 (1035/4233), and in order to provide a 'notional' number of normal embryos corresponding to this sample, the numbers of normal embryos in the four age groups (as in **Table 2**) were scaled down by this fraction. Assuming that the sampling could be regarded as random, this device provided a theoretical sub-population from which the incidences of the individual abnormalities could be estimated. The resulting summaries are presented in **Table 3**.

## Results

### PGD versus re-analysis data

A total of 6054 embryos that had PGD results were included in this study. Of those, 1132 (18.7%) were re-analysed, all classified by PGD as abnormal. Of those, after re-analysis 97 (8.6%) were either normal or mosaics with less than 3/8 abnormal cells, and were considered false positive errors. However, this should be considered a mean, as accuracy has improved over the years. Since 2004, in which a third hybridization was used with another probe binding to the locus of the chromosome showing doubtful results, the false negative error rate has decreased significantly from 11.7 (82/699) to 3.5% (15/433) ( $P < 0.001$ ).

The errors occurred randomly irrespective of morphology, with group 4 error having 9.3% false negatives in the re-analysed embryos (61/658), and the rest of groups having 7.6% (36/474) (difference not statistically significant). For the purposes of this study and because of the randomness of false positive errors, it was considered appropriate to use the re-analysis data for those embryos that had it, and to use PGD data for those that did not.

### Frequency of normal embryos according to maternal age, and developmental/morphological groups

The proportions of normal embryos, classified by maternal age and morphological group, are displayed in **Table 2**. Both maternal age and development/morphology parameters had a very large impact on the proportion of normal embryos, but there was no evidence of interaction, so that these factors appeared to act independently; that is, the age effect was fairly constant over morphological and developmental groups, and *vice versa*. The age effect on aneuploidy was very pronounced ( $P < 0.001$ ). The main deviation in developmental/morphological groups was between group 4 and the remaining three groups, possessing various imperfections ( $P < 0.001$ ). For the embryos with the best morphology and development and most likely to be replaced (group 4), only 44% of embryos from patients younger than 35 were chromosomally normal, decreasing to 21% in patients 41 and older. In contrast, group 1, the group with the worst morphology and development, had only 30% normal embryos from patients younger than 35, and 12% in embryos from patients 41 and older.

### Types of abnormalities by maternal age

In order to make reliable inferences about the effect of age on individual genetic abnormalities, it must be assumed that the re-analysis was carried out in a fairly random fashion, and that no bias was inadvertently introduced. The average age and standard error of the group of embryos re-analysed was  $38.9 \pm 0.106$ , and for those not re-analysed the mean value and standard error was  $38.8 \pm 0.064$ . Thus, for this important parameter of the

age distribution, the values were almost identical. In addition, a total of 18% (658/3571) embryos from group 4, 20% (145/721) of group 3, 18% (269/1516) of group 2, and 25% (61/245) of group 4 were re-analysed, so it could reasonably be assumed there was no bias in the morphological types of embryos. It is not desirable to attach too much emphasis to the precise and absolute values of the incidences displayed in **Table 3**, since the totals on which they were based were themselves estimated under the assumptions noted.

The changes in incidences by age group are likely to be quite reliable, however, and therefore provide a useful summary of the relationship between the various abnormalities and age.

The notional frequencies of normal embryos to be matched with the re-analysed abnormal embryos were 116, 119, 128 and 82 respectively for the four age groups. Using these figures to generate notional totals for the number of embryos, the incidence of the individual abnormalities for the age groups may be estimated. Thus, the figure of 9.5% in the first cell of **Table 3** means that the probability of obtaining an aneuploid embryo in this age group is estimated as 0.095, with a similar interpretation for the other cells. The *P*-values in **Table 3** refer to tests for variation in the incidences across the age groups. Thus, the change from 9.5% aneuploidy in the first group to 16.3% in the highest age group was statistically significant (*P* < 0.01).

**Table 3** is a complete summary of the incidences of individual chromosome abnormalities detected after re-analysis. In total, 19.6% of abnormal embryos were purely aneuploid, 31.8% were aneuploid together with other post-meiotic abnormalities (polyploidy, haploidy, extensive mosaicism), and 48.6% had only post-meiotic abnormalities.

The content of **Table 3** provides reasonably conclusive evidence (*P* < 0.001) that aneuploidy increased with advancing age of the patient, while post-meiotic abnormalities (extensive mosaicism, haploidy, polyploidy) did not, as there was no corresponding increase.

Of the 532 aneuploid embryos confirmed as aneuploid after re-analysis, 378 had one chromosome aneuploid in all or most cells, 127 had two aneuploid chromosomes (double aneuploidy), 21 had three, five had four, and one had five. In total, there were 720 aneuploidy events. Of those, chromosome 16 was involved in 154 events, chromosome 22 in 144, chromosome 21 in 116, chromosome 15 in 95, chromosome 18 in 73, chromosome 13 in 68, chromosome 17 in 40, and XY in 30.

### Chromosome abnormalities and indication for PGD

Advanced reproductive maternal age (AMA) is considered here as 38 years and above (*n* = 3531 cycles). Patients under 38 had the following indications: 723 cycles were from patients with recurrent pregnancy loss (RPL), 480 with recurrent implantation failure (RIF), 305 with a previous aneuploid pregnancy (ANE), and 1015 with none of the above indications (Other). These latter groups were mostly patients 35–37, for which PGD is offered, and younger ones that requested the test themselves.

**Table 4** summarizes the chromosomes found both per indication and in relation to embryo morphology and development. Overall, most indications had similar frequencies of chromosomally normal type 4 embryos (41–44%), with the exception of advanced maternal age (25%) (*P* < 0.001). However, when the embryos

**Table 4.** Chromosome abnormalities by embryo type and preimplantation genetic diagnosis indication.

Embryo type	Indication	Normal	Abnormal	Total %	Normal
Type 1 to 3	AMA	333	1132	1465	23 <sup>c</sup>
	ANE	38	74	112	34 <sup>a</sup>
	Other	153	277	430	36 <sup>a</sup>
	RIF	52	151	203	26 <sup>b</sup>
	RPL	75	198	273	27 <sup>b</sup>
Type 4	AMA	526	1540	2066	25 <sup>d</sup>
	ANE	82	111	193	42 <sup>c</sup>
	Other	255	330	585	44 <sup>c</sup>
	RIF	113	164	277	41 <sup>c</sup>
	RPL	194	256	450	43 <sup>c</sup>
Total	AMA	859	2672	3531	24 <sup>f</sup>
	ANE	120	185	305	39 <sup>g</sup>
	Other	408	607	1015	40 <sup>g</sup>
	RIF	165	315	480	34 <sup>g</sup>
	RPL	269	454	723	37 <sup>g</sup>
	Total	1821	4233	6054	30

AMA = advanced maternal age; ANE = previous aneuploid conception; RIF = recurrent implantation failure; RPL = recurrent pregnancy loss; Other = none of the above.

<sup>a</sup>–<sup>f</sup>Denote significant differences: a versus c, *P* < 0.001; a versus b, *P* < 0.01; d versus e and f versus g, *P* < 0.001.

were morphologically and/or developmentally abnormal, RIF and RPL patients had a frequency of chromosomally normal embryos (26 and 27% respectively) similar to AMA patients (23%), and lower than ANE (34%) ( $P < 0.05$ ) and Other (36%) ( $P < 0.01$ ).

## Discussion

The present study attempted to re-evaluate previous studies that had described the relationship between chromosome abnormalities, embryo morphology, and maternal age (Munné *et al.*, 1995, 2004a, 2006; Márquez *et al.*, 2000; Magli *et al.*, 2001; Bielanska *et al.*, 2002a,b; Gianaroli *et al.*, 2007; Munné, 2006). Using data from embryos analysed by PGD only, it was possible to study most of the cohort of embryos produced, with the exception of embryos with three or fewer cells. In previous studies, morphologically and developmentally normal embryos were seldom analysed with PGD. Although PGD can only study one cell, this is enough to determine, with the present 9% error rate (3.5% with more recent technology), if an embryo is normal or abnormal. Thereafter, re-analysis of all cells of abnormal embryos permits the exact chromosome abnormality to be ascertained with little doubt. The analysis of more than 6000 embryos for eight chromosome pairs makes this study the most extensive and comprehensive to date on chromosome abnormalities in human cleavage stage embryos.

The results confirm previous trends (Munné *et al.*, 1995; Márquez *et al.*, 2000), such as that aneuploidy increases in cleavage stage embryos with maternal age, irrespective of embryo morphology, as it does not increase with decreasing developmental competence. Also confirmed were the previous observations (Munné *et al.*, 1995, 2006; Márquez *et al.*, 2000; Magli *et al.*, 2001, 2007; Bielanska *et al.* 2002a,b; Munné, 2006) that post-meiotic abnormalities such as mosaicism, polyploidy and haploidy increase with decreasing embryo development and with increasing dysmorphism (multinucleation, fragmentation, unevenness); but not with maternal age. Also confirmed is the fact that post-meiotic abnormalities are the most frequent type of chromosome abnormality, and not aneuploidy. Thus, it is clear that maternal age is only one of the causes of chromosome abnormalities.

What has not been well described heretofore has been the extent of chromosome abnormalities in the embryos with the highest scores of morphology and development rate. The present study indicates that only 44% of embryos from young patients are normal for the chromosomes studied, and this frequency decreases with advancing maternal age, to 42 (35–37 years), 30 (38–40 years), and 21% in patients aged 41 and older. A recent study by Magli *et al.* (2007), also analysing similar material, similarly reported close to 50% rates of chromosome abnormalities in embryos with the best morphology. Studies using spectral karyotyping, or comparative genome hybridization (CGH), although very limited in the number of embryos tested so far (Sandalinas *et al.*, 2002; Voullaire *et al.*, 2002; Wilton *et al.*, 2003; Gutierrez-Mateo *et al.*, 2004, 2005), usually indicate that about 70% of all abnormalities can be detected with the eight chromosome probes used in PGD, so the frequency rates determined for euploidy (chromosomally normal) in developmentally and morphologically normal embryos are actually probably lower at 31, 29, 21 and 15% respectively.

It could be argued that the present study has the limitation that patients indicated for PGD and of young age had either the indication of RIF (average age 34.5 years), ANE (34.4 years) or of RPL (34.7 years). It is known that patients with ANE produce more aneuploid embryos than other patients (Munné *et al.*, 2004b), and that RIF patients have more chromosome abnormalities overall than other groups of patients (Gianaroli *et al.*, 1999, 2001; Kahraman *et al.*, 2000; Pehlivan *et al.*, 2002). In contrast RPL patients, although fertile, produce rates of chromosome abnormalities that are similar to or higher than those found in non-RPL infertile patients (Simon *et al.*, 1998; Rubio *et al.*, 2003; Munné *et al.*, 2005). Thus, the young group of patients here included may be biased towards producing more chromosome abnormalities than the typical young infertile couple. However, egg donation cycles (Munné *et al.*, 2006) with PGD also indicate rates of chromosome abnormalities similar (57%) to those reported here.

The other age groups were almost all patients indicated for a maternal age of 35 and over, so there was no possibility of the above bias.

Interestingly, all indications other than AMA had the same rate of chromosomally normal type 4 embryos (41–44%), showing that either (a) appropriate indications have been used; or that (b) the rate of chromosome abnormalities is independent of indication in patients under 38. The group Other were mostly patients aged 35–37 with age as their only indication, so this already suggests that (b) is the most likely explanation. However, if PGD were offered to patients under 35 and sufficient results were obtained, a comparison with the older group would be very interesting.

In summary, taking into account only patients 35 and older, the total rates of chromosomally normal embryos estimated (29, 21, 15%) are close to the maximum implantation potential described for these embryos when morphological selection is performed, indicating once again that the major cause of embryos not reaching term is chromosome abnormalities.

The present data, at least for patients 35 and older, have the value of providing potentially important information for practitioners to counsel their patients about the risk of chromosome abnormalities that a specific embryo may have, depending on its maternal age and morphology.

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