

## Articles

# Chromosome mosaicism in cleavage-stage human embryos: evidence of a maternal age effect



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Santiago Munné has been director of PGD at Saint Barnabas Medical Center since 1995. 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 100 PGD cycles for translocations and over 600 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

The present study evaluated mosaicism in a large series of cleavage-stage human embryos analysed by fluorescence in-situ hybridization. Only embryos with at least three cells analysed were included ( $n = 1235$ ), of which 556 were mosaics. The most common types of mosaicism were chaotic (48%), diploid/polyploid (26%), and those caused by mitotic non-disjunction (25%). The number of abnormal cells per embryo ranged from 44% in diploid/polyploid to 84% in chaotic mosaics. Chromosome 16 was most commonly involved in mitotic non-disjunction mosaics. While overall mosaicism did not increase with maternal age, the average maternal age of the embryos that had mosaics caused by mitotic non-disjunction was significantly higher than that for normal or other mosaic embryos ( $P < 0.001$ ). During the cleavage stage, the embryonic genome is not yet fully activated and consequently the mRNA and protein pools are still similar to those found in the oocyte. We therefore propose that the malfunctioning of the meiosis apparatus, which is similar to the mitotic one, may cause either meiotic errors or mitotic non-disjunction at cleavage-stage embryo development.

**Keywords:** IVF, maternal age, mitotic non-disjunction, preimplantation genetic diagnosis, uniparental disomy 16

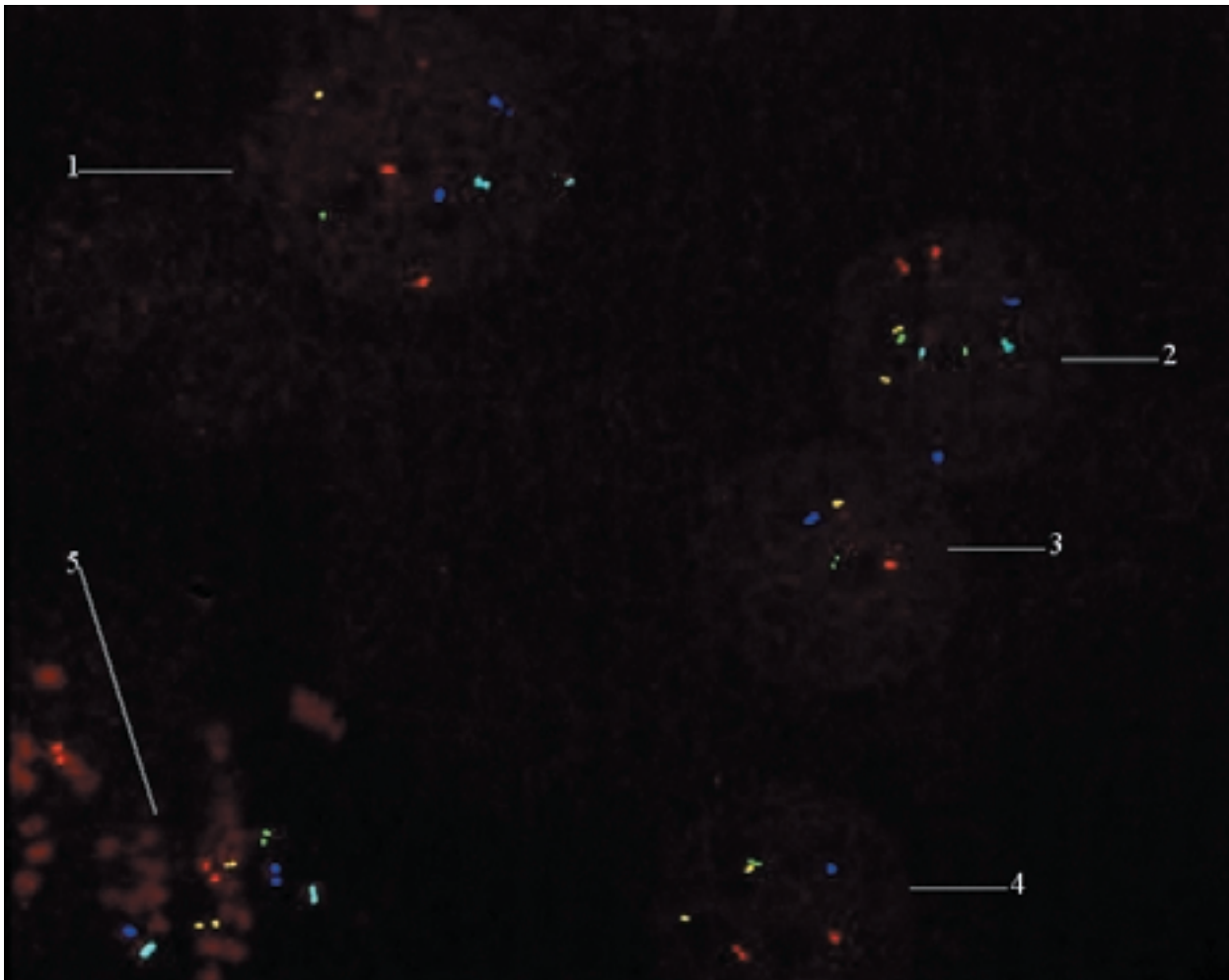
### Introduction

It is estimated that 10–30% of IVF cleavage-stage embryos are mosaic (Munné *et al.*, 1994a, 1995; Pellestor *et al.*, 1995; Delhanty *et al.*, 1997). While the rate of mosaicism detected by karyotyping was considered an underestimation based on the analysis of only one or two cells per embryo (Almeida and Bolton, 1996), the high rate of mosaicism found after fluorescence in-situ hybridization (FISH) may be an artefact. Strict classification criteria for FISH have consistently found the same high rates (Munné and Cohen, 1998; Márquez *et al.*, 2000). The rate of preimplantation mosaicism appears extremely high compared with the 1–2% found in prenatal diagnosis (Kalousek *et al.*, 1991). Relevance of FISH data was questioned because the embryos analysed by FISH were cultured in-vitro and obtained from infertile patients given hormones to produce ovarian stimulation for IVF treatment. Among other things, the culture conditions and regimes of

hormonal stimulation can increase the frequency of mosaicism (Munné *et al.*, 1997) and, as a consequence, embryos generated by IVF procedures may not fully reflect the situation in natural cycles.

The types of mosaics observed in cleavage-stage embryos are also more diverse than those observed in spontaneous abortions, indicating that some mosaic embryos or some cell lines are incompatible with later stages of embryo development. Cleavage-stage mosaic embryos have been classified according to their overall ploidy (haploid, diploid, or polyploid mosaics) and mechanism of formation (mitotic non-disjunction, anaphase lag, endoreduplication, diploid/polyploid mosaics) (Munné and Cohen, 1998). Embryos are defined as chaotic (**Figure 1**) when the mechanism of mosaicism formation is either unclear or random (Harper *et al.*, 1995; Harper and Delhanty, 1996; Delhanty *et al.*, 1997). In a recent review of mosaicism, three





**Figure 1.** FISH on a chaotic embryo using probes for chromosomes 13 (red), 16 (light blue), 18 (dark blue), 21 (green), 22 (yellow). Nucleus 1 shows two signals for chromosome 13, two for chromosome 16, two for chromosome 18, one for chromosome 21 and one for chromosome 22 or for short, 2[13],2[16],2[18],1[21],1[22]; nucleus 2 has 2[13],2[16],2[18],2[21],2[22]; nucleus 3 has 1[13],0[16],1[18],1[21],1[22]; nucleus 4 has 2[13],0[16],1[18],1[21],2[22] and nucleus 5, a metaphase, has 2[13],2[16],2[18],1[21],2[22].

frequent categories of mosaics were identified: (i) chaotic mosaics (47% of all mosaics), (ii) diploid/polyploid mosaics (28%), and (iii) diploid/aneuploid mosaics (19%); anaphase lag and endoreduplication mosaics were rarely identified (Munné and Cohen, 1998).

The mechanisms of formation of the three main groups of mosaics are diverse:

(i) Diploid/polyploidy mosaics (2N/pol) are mostly composed of a diploid and a tetraploid cell line, but may also contain triploid, hexaploid, octoploid, haploid, and other ploidies. These early phenomena are not necessarily associated with the normal physiological formation of polyploid cells in trophoblastic cells (Barlow *et al.*, 1972; Angell *et al.*, 1989; Benkhalifa *et al.*, 1993; Evsikov and Verlinsky, 1998; Sandalinas *et al.*, 2001). In contrast, haploid cells could be caused by nuclear division without previous chromosome duplication, or by a polar body nucleus incorporated into the zygote. Triploid cell lines may be either caused by endoreduplication of one of the chromosome sets (Munné *et al.*,

1994a,b), by the incorporation of a second spermatozoon (Tuerlings *et al.*, 1993), or a polar body (Muller *et al.*, 1993) into a cell line.

(ii) Chaotic embryos, first described by Delhanty *et al.* (1997), seemed the most common type of mosaic, but little is known about their formation mechanism. Delhanty *et al.* (1997) stated that it is a patient-related phenomenon.

(iii) Mitotic non-disjunction is the third most common mechanism of mosaicism (hereinafter referred to as 'mitotic aneuploid' mosaics) in cleavage-stage embryos, and it was described for all chromosomes analysed (Munné *et al.*, 1994a, 1995, 1998a,b; Harper and Delhanty, 1996; Delhanty *et al.*, 1997; Evsikov and Verlinsky, 1998; Munné and Cohen, 1998; Bahçe *et al.*, 2000; Márquez *et al.*, 2000). Little expression of the embryonic genome is seen until the four- to eight-cell stage on day 3. Consequently, chromosomally abnormal cells may not be selected against until the morula stage (Sandalinas *et al.*, 2001). These mosaics are unlikely to be the result of self-correction of meiotic aneuploidy of the subsequent cell line.

**Table 1.** Classification of mosaic embryos.

a) Classification according to formation mechanism:

Type	Characteristics
Diploid mosaics:	Overall ploidy 2N
(i) Chaotic	All or most abnormal cell having a different chromosome count
(ii) 2N/pol	A diploid cell line and one or several other lines with other ploidies
(iii) Mitotic aneuploid:	
Mitotic non-disjunction	Usually with a normal, monosomic and trisomic cell lines
Mitotic anaphase lag	>20% monosomic for one chromosome and the rest normal
Endoreduplication	Abnormal cells with one chromosome presenting multiple copies
(iv) Split	Abnormal cell lines created by cell division without previous DNA replication
Polyplod mosaics	Overall ploidy $\geq 3N$
Haploid mosaics	Overall ploidy $\leq N$

b) Classification according to impact on embryo and fetal development:

Type	Characteristics
Benign	Mosaics with less than 3/8 of their cells being abnormal with the exception of mitotic aneuploid mosaics
Detrimental	Any mitotic aneuploid mosaic Mosaics with $\geq 3/8$ of their cells being abnormal

Furthermore, these mosaics usually show disomic, trisomic, and monosomic cell lines, which indicates a mitotic non-disjunction origin.

Overall, mosaicism in cleavage-stage embryos does not increase with maternal age (Munné and Cohen, 1998), but the effect of maternal age has not been studied in relation to type of mosaic, and specifically in relation to mitotic aneuploid mosaics. Sex chromosome mosaicism detected by amniocentesis does not increase with maternal age (Ferguson-Smith and Yates, 1984). Similarly, for most chromosomes studied, aneuploidy detected in fetuses and adults and generated by mitotic mosaicism does not increase with maternal age (Antonarakis *et al.*, 1993; Sherman *et al.*, 1994; Wolstenholme, 1995; Eggermann *et al.*, 1996; Lamb *et al.*, 1996; Robinson *et al.*, 1996), except maternal trisomy X (Thomas *et al.*, 2001).

**Table 2.** Overall rates of normal and abnormal embryos according to maternal age (years).

	$\leq 34.9$	35–39.9	$\geq 40$	Total
Normal (%)	243 (42.8)	301 (41.1)	211(34.9)	755 (39.7)
Haploid	16	24	14	54
Polyplod	48	57	41	146
Aneuploid	59	109	151	319
Aneuploid and mosaic	10	28	36	74
Mosaic benign	56	34	19	109
Mosaic detrimental	118	141	114	373
Abnormal not reanalysed <sup>c</sup>	17	38	18	73
Total	567	732	604	1903
Total aneuploid (%) <sup>d</sup>	69(12.2) <sup>a</sup>	137(18.7) <sup>a</sup>	187(31.0) <sup>a</sup>	393(20.6)
Total mosaic (%) <sup>e</sup>	184(32.4) <sup>b</sup>	203(27.7) <sup>b</sup>	169(28.0) <sup>b</sup>	556(29.2)

<sup>a</sup> Values significantly different ( $P < 0.001$ ).<sup>b</sup> Values not statistically significant.<sup>c</sup> Only one or two cells analysed and abnormality not consistent with single or double aneuploidy, haploidy, or polyplod. Probably a chaotic mosaic but not enough cells analysed to confirm it.<sup>d</sup> Aneuploid + aneuploid and mosaic.<sup>e</sup> Mosaic detrimental + mosaic benign + aneuploid and mosaic.

The present study evaluated 1235 cleavage-stage embryos analysed with FISH to review prior assumptions (Munné *et al.*, 1995; Márquez *et al.*, 2000) including (i) lack of a maternal age effect, (ii) mechanism of mosaicism, (iii) cleavage onset, and (iv) impact on preimplantation genetic diagnosis. We observed that mitotic non-disjunction, but not other mosaic types, increased with maternal age.

## Materials and methods

Embryos used for this project were donated for research in accordance with guidelines approved by the local Internal Review Board, including informed written consent in each case. The largest group of embryos was considered morphologically or developmentally unsuitable for transfer or cryopreservation (Alikani *et al.*, 2000). The others were classified as chromosomally or developmentally abnormal after preimplantation genetic diagnosis (PGD). Embryos classified by PGD as chromosomally abnormal usually had all or most of their cells analysed after PGD, while those classified as normal were usually replaced back to the patient. PGD patients therefore normally had all their embryos analysed, although not all embryos were analysed fully.

Non-replaced embryos were disaggregated and all cells fixed

**Table 3.** Mosaic types, frequencies, and average maternal age.

Type of mosaic	n (%)	Mean % cells analysed	Mean % abnormal cells	Mean maternal age (years)
<b>Chaotic</b>				
Total chaotic	266 (47.8)	6.3	84.3 <sup>b</sup>	36.8 <sup>a</sup>
2N/pol <sup>c</sup>				
2N/4N	76	7.0	37.5	36.5
2N/3N	21 <sup>e</sup>	5.6	51.8	35.9
2N/N	15	6.7	37.1	35.6
2N/other N	15	5.1	37.1	35.6
2N/4N/6N	8	6.6	53.5	38.7
2N/4N/N	5	9.0	37.1	35.6
2N/4N/8N	4	5.5	59.8	36.7
Total 2N/pol	144(25.9)	6.6	43.7 <sup>b</sup>	36.3 <sup>a</sup>
<b>Mitotic aneuploid</b>				
Non-disjunction (only mosaic)	96	8.7	65.3	38.0
Meiotic and mitotic non-disjunction <sup>d</sup>	12	9.8	72.8	38.5
Anaphase lag	28	6.1	65.7	37.8
Endoreduplication	2	7.5	14.0	41.8
Total mitotic aneuploid	138 (24.8)	8.2	65.3 <sup>b</sup>	38.1 <sup>a</sup>
<b>Split</b>				
Total split	8 (1.4)	7.2	53.9 <sup>b</sup>	34.9 <sup>a</sup>

<sup>a,b</sup> Values with same superscript were significantly different ( $P < 0.001$ ).

<sup>c</sup>Other mosaic types may also have had polyploid cell lines but were not counted in the 2N/pol group.

<sup>d</sup>These were embryos fully trisomic or monosomic for a specific chromosome and in addition they were mosaic for at least that same chromosome.

<sup>e</sup>One was a sex chimera 2N, XX/3N XXY mosaic.

individually following previous published protocols (Munné *et al.*, 1998b). All embryos were analysed for chromosomes X, Y, 13, 18, and 21. Some were also analysed for chromosome 16, and some with additional probes for chromosomes 15, 16, and 21. All used previously published FISH protocols (Munné *et al.*, 1995, 1998b; Bahçe *et al.*, 2000).

For the evaluation of mosaic data, only embryos with at least three cells analysed were included.

To classify the different chromosome abnormalities detected by FISH, the following criteria were used: normal, aneuploid, haploid, and polyploid embryos were those where all the cells of the embryo had the same chromosome constitution or <10% differing cells. The 10% cut-off is included here because it is the approximate FISH error level.

Diploid mosaic embryos were those mosaics that had a diploid line; or, on average, the number of chromosomes per cell was diploid. Similarly, when the number of diploid cells in a mostly haploid embryo was less than 29% (1/4, 1/5, 1/6, 2/7,

2/9, 2/10 cells) the embryo was classified as haploid mosaic, and when a mostly polyploid embryo had less than 29% diploid cells it was classified as polyploid mosaic. However, to simplify the analysis, polyploid and haploid mosaics were grouped with pure polyploid and haploid embryos and were not further analysed for mosaicism. This study focused only on diploid mosaic embryos because they usually contain diploid cell lines and can arrive to blastocyst stage (Sandalinas *et al.*, 2001).

Some embryos showed chaotic chromosome complements. Although some researchers (Harper *et al.*, 1995; Harper and Delhanty, 1996) consider such embryos as a separate mosaic category, these embryos were counted as diploid mosaics if on average they had a diploid chromosome content. Throughout, the scoring criteria of Munné and Weier (1996) were used to differentiate FISH errors from mosaicism.

Mosaics embryos were classified into different sub-groups (Table 1) to simplify the analysis. Diploid mosaic embryos were classified into four groups: (i) mosaics with a diploid and

**Table 4.** Examples of mosaic embryos.

Mosaic type/subtype	No. cells	Chromosome count						
		XY	13	15	16	18	21	22
2N/pol		XY	13	15	16	18	21	22
2N/4N	6	XY	2	2	2	2	2	2
	2	XXYY	4	4	4	4	4	4
2N/3N (sex chimera)	3	XX	2	2	2	2	2	2
	3	XXY	3	3	3	3	3	3
2N/other	4	XY	2	2	2	2	2	2
	2	XXYY	4	4	4	4	4	4
	1	XXY	3	3	3	3	3	3
	1	3X3Y	6	6	6	6	6	6
	1	6X2Y	8	8	7	8	8	7 <sup>a</sup>
Chaotic	2	XX	2	2	2	2	2	2
	1	X	2	2	1	3	1	2
	1	XX	2	1	1	2	3	3
	1	X	0	1	2	3	0	0
	1	XXX	3	3	2	0	2	2
	1	XXX	3	4	1	5	4	3
Split	3	XX	2	na	2	2	2	na
	3	XXX	2	na	2	3	0	na
	2	X	2	na	2	1	4	na
Mitotic aneuploid								
Anaphase lag	6	XY	2	2	2	2	2	2
(of chromosome 16)	4	XY	2	2	1	2	2	2
Endoreduplication	6	XY	2	na	2	2	2	na
(of chromosome 18)	1	XY	2	na	2	7	2	na
Non-disjunction	3	XY	2	2	2	2	2	2
(of chromosome 21)	1	XXYY	4	4	4	4	2	4
	2	XY	2	2	2	2	1	2
	1	XY	2	2	2	2	3	2
Meiotic (trisomy 16) and mitotic non-disjunction	3	XX	2	2	3	2	2	2
(of chromosome 16)	2	XX	2	2	2	2	2	2
	1	XX	2	2	3	2	2	1 <sup>b</sup>
	1	XX	2	2	4	2	2	2
	1	XXXX	4	4	6	4	4	4
Meiotic (monosomy 22) and mitotic non-disjunction	5	XX	2	2	2	2	2	1
(of chromosome 22)	2	XX	2	2	2	2	2	0
	1	XX	2	2	2	2	2	2

na: not analysed.

<sup>a</sup>Chromosomes 16 and 22, with seven copies are considered FISH errors (should have eight copies).

<sup>b</sup>Chromosome 22, with only one copy, is considered a FISH error (should have two copies).

**Table 5.** Onset of mosaicism. Values are percentages unless otherwise indicated.

Onset of mosaicism	1st division	2nd division	3rd division	Total no. cells
Abnormal cells	75–100	74–38	>38	
All diploid mosaics	51	26	24	556 <sup>a</sup>
2N/pol	15	33	51	144 <sup>a,c</sup>
Chaotic	73	18	9	266 <sup>a,d</sup>
Mitotic aneuploid	45	33	22	138 <sup>a,c,d</sup>
Split	37	26	37	8 <sup>a</sup>
Polyploid mosaic	73	22	4	49 <sup>b</sup>
Haploid mosaic	80	8	12	25 <sup>b</sup>
Dispermic	69	30	0	36 <sup>b</sup>

<sup>a</sup>This study.

<sup>b</sup>Munné and Cohen (1998).

<sup>c,d</sup> Values with different superscripts were significantly different ( $P < 0.001$ ).

**Table 6.** Chromosome abnormalities analysed by maternal age and according to whether PGD was carried out or not. Numbers of patients are given in parentheses.

Type	PGD	No PGD	Total
Mosaic: 2N/POL	37.8 ± 0.44 (61)	35.3 ± 0.43 (83)	36.4 ± 3.9 (144)
Mosaic: mitotic aneuploid	38.7 ± 0.34 (87) <sup>a</sup>	36.9 ± 0.57 (51) <sup>d</sup>	38.0 ± 3.6 (138) <sup>g</sup>
Mosaic: chaotic	36.9 ± 0.39 (118)	36.7 ± 0.35 (148)	36.8 ± 4.2 (266)
Mosaic: split	35.7 ± 2.51 (4)	34.0 ± 2.30 (4)	34.9 ± 4.6 (8)
Abnormal not reanalysed	37.3 ± 0.42 (70)	36.9 ± 0.64 (3)	37.3 ± 3.4 (73)
Aneuploid <sup>j</sup>	38.8 ± 0.20 (253) <sup>b</sup>	37.5 ± 0.51 (66) <sup>e</sup>	38.5 ± 3.4 (319) <sup>h</sup>
Haploid	37.9 ± 0.66 (33)	35.5 ± 0.86 (21)	37.0 ± 4.0 (54)
Normal	37.4 ± 0.16 (527) <sup>c</sup>	35.6 ± 0.25 (228) <sup>f</sup>	36.8 ± 3.8 (755) <sup>i</sup>
Polyploid	37.4 ± 0.44 (75)	35.9 ± 0.42 (71)	36.7 ± 3.8 (146)
Total	1228	675	1903

Statistical comparisons: a versus c,  $P < 0.05$ ; d versus f,  $P < 0.01$ ; b versus c, e versus f, g versus i, h versus i,  $P < 0.001$ .

<sup>j</sup>The 74 aneuploid and mosaic embryos from **Table 2** are here included in their respective mosaic groups.

one or more polyploid cell lines (2n/pol mosaics); (ii) mosaics usually with each abnormal cell having a different chromosome count than the other (chaotic); (iii) mosaics created either by mitotic non-disjunction, mitotic anaphase lag, or mitotic endo-reduplication of one or few chromosomes (mitotic aneuploid mosaics); (iv) mosaics created by cellular division without previous chromosome replication creating two cell lines that complement each other (split mosaics).

As in previous studies, mosaics were classified as limited if they had <3/8 of their cells abnormal; or extensive if they had ≥3/8 cells abnormal. Limited mosaics were considered benign and mostly normal: extensive mosaics were considered detrimental for embryo survival based on freeze-thawing (Tarin *et al.*, 1992) and blastocyst data (Sandalinis *et al.*, 2001). In addition, while haploid, chaotic, split and polyploid cell lines usually arrest (Sandalinis *et al.*, 2001), trisomic cell lines may arrive to term. Thus, limited mosaics containing aneuploid cells may have serious results for the developing fetus. For this reason, mosaic embryos were classified as benign or detrimental. Benign embryos were limited mosaics with the exception of mitotic aneuploid mosaics; and detrimental embryos were extensive mosaics in addition to any aneuploid mosaic.

## Statistical analysis

Association between maternal age and the main groupings of abnormalities, or of sub-groupings within the main abnormality groups, was investigated by carrying out analyses of variance (ANOVA) on the maternal ages. In addition to the one-way ANOVA carried out to investigate differences between the main groupings (2N/pol, chaotic, mitotic aneuploid, and total split), further one-way ANOVAs were carried out to investigate systematic differences between sub-groups within the main groups 2N/pol and mitotic aneuploid.

## Results

### Embryo population, mosaic types and frequencies

All embryos ( $n = 1903$ ) were analysed for chromosomes X, Y, 13, 18, and 21. Of these embryos, 321 embryos were additionally analysed for chromosome 16, and 1026 embryos additionally for chromosomes 15, 16, and 22. Of the embryos studied here, 675 were considered unsuitable for transfer from non-PGD cycles and 1228 were obtained from PGD cycles. In total, 755 (39.7%) were chromosomally normal, and the rest aneuploid, polyploid, haploid or mosaic (**Table 2**).

Not all embryos were fully re-analysed. Embryos classified as normal after PGD and with good development and morphology were usually replaced in the patient, while others, even when classified by PGD as abnormal, could not be re-analysed either because they degenerated before analysis could be done, or for technical and time constraints.

Embryos were classified as mosaics only when three or more cells had been analysed. Overall, 556 embryos with more than three cells analysed were mosaics, that is 29.2% of all embryos or 45.0% (556/1235) of embryos with three or more cells analysed.

Embryos classified as aneuploid based on one ( $n = 94$ ) or more cells ( $n = 304$ ) showed that aneuploidy for the chromosomes analysed increased significantly with maternal age, from 12.2% in embryos from patients aged <35 years to 31.0% in embryos from patients aged 40 years or older ( $P < 0.001$ ) (**Table 2**). Similarly, the proportion of chromosomally normal embryos decreased with maternal age, from 42.8% to 34.9% in the same maternal age groups (**Table 2**). In contrast, mosaicism as a whole was not affected by maternal age, being constant around 28–32% in all three maternal age groups.

**Table 3** lists different categories of mosaicism. Chaotic mosaics were the most common type, nearly 50% of all cases. Almost all of the remaining mosaics were cases of 2N/pol and mitotic aneuploids, with split mosaics identified in only 1.4% of cases. Most mitotic aneuploid mosaics were caused by mitotic non-disjunction, followed by anaphase lag and endoreduplication. In addition, 12 of the mosaics that had mitotic non-disjunction were fully aneuploid. For example, meiotic non-disjunction, associated with mitotic non-disjunction was indicated when an embryo was mostly trisomic for chromosome 16, with some cells normal and others tetrasomic for chromosome 16 (**Table 4**).

### Mosaic types and average abnormal cells

As shown in **Table 3**, 2N/pol mosaics had the lowest percentage of abnormal cells (44%), while chaotic mosaics had an average of 84% ( $P < 0.001$ ). The percentage of abnormal cells is a rough indicator of the cell division origin of the mosaicism, with most cells being abnormal (100–75% or 8/8–6/8 of the cells), indicating a first division origin, about half of the cells (74–38% or 6/8–3/8 of the cells) being abnormal, indicating a second division origin, and about one quarter of abnormal cells (10–37% or less than 3/8 of the cells) indicating a third or later division origin. The results indicated that in 50.7% of mosaic embryos the abnormality originated at the first mitotic division; 25.7% at the second; and 23.6% at the third or later. However, 2N/pol mosaics seldom originated at the first mitotic division (15%) while chaotic and mitotic aneuploid mosaics originated mostly at the first mitotic division (73.3 and 44.9% respectively) ( $P < 0.001$ ) (**Table 5**).

To calculate which chromosomes were producing mitotic aneuploid mosaicism, it was necessary to control for the chromosomes analysed because not all embryos were analysed with the same number of probes. Of the 138 mitotic aneuploid mosaics, all were analysed for XY, 13, 18 and 21 chromosomes; 110 were also analysed for chromosome 16; and 90 also for chromosomes 15 and 22. Chromosome involvement was XY, 19% ( $n = 26$ ); 13, 22% ( $n = 31$ ); 15, 18% ( $n = 16$ ); 16, 37% ( $n = 41$ ); 18, 21% ( $n = 29$ ); 21, 25% ( $n = 35$ ); and 22, 21% ( $n = 19$ ). The rate of mitotic aneuploid mosaicism produced by chromosome 16 was significantly higher than that produced by chromosome 15 and XY ( $P < 0.005$ ), 18 ( $P < 0.01$ ), 13 and 22 ( $P < 0.05$ ).

### Mosaic types and average maternal and paternal age

Mosaicism, as a whole, does not increase with maternal age. However, when the four types of mosaics are taken independently, there was strong evidence of systematic

**Table 7.** Statistical analysis of maternal and paternal age.

Type of embryo	Maternal age (years)	Paternal age (years)
Mosaic 2N/pol	36.3 ± 0.32	38.7 ± 0.47
Mosaic chaotic	36.8 ± 0.26	39.8 ± 0.47
Mosaic mitotic aneuploid	38.1 ± 0.31	40.4 ± 0.57
Mosaic split	34.9 ± 1.61	35.0 ± 1.38
P-value	<0.001	0.07

**Table 8.** Risk of PGD misdiagnosis due to mosaicism.*Risk of classifying an abnormal embryo as normal*

	Overall % (frequency) (A)	% normal cells (B)	Risk of misdiagnosis (%) (AxB)
2N/POL (detrimental)	3.7 (70/1903)	34.8	1.3
Chaotic (detrimental)	12.7 (242/1903)	9.8	1.2
Split (detrimental)	0.3 (5/1903)	29.8	0.1
Mitotic aneuploid (all)	6.6 (126/1903)	24.2	1.6
Meiotic and mitotic aneuploid (all) <sup>a</sup>	0.6 (12/1903)	12.2	0.1
Total	23.9 (455/1903)	18.0	4.3

*Risk of classifying a mostly normal embryo as abnormal:*

	Overall % (frequency) (A)	% abnormal cells (B)	Risk of misdiagnosis (%) (AxB)
2N/POL (benign)	3.9 (74/1903)	23.1	0.9
Chaotic (benign)	1.3 (24/1903)	24.9	0.3
Split (benign)	0.2 (3/1903)	26.7	0.1
Total	1.3		
Total misdiagnosis rate due to mosaicism		5.6	

<sup>a</sup>These were embryos fully trisomic or monosomic for a specific chromosome and in addition mosaic for the same chromosome.**Table 9.** Accuracy of PGD results based on embryo re-analysis.

PGD diagnosis	Re-analysis	No. embryos
Normal	Normal	353
Normal	Mosaic benign	6
Normal	Mosaic detrimental	8 <sup>a</sup>
Normal	Aneuploid	5 <sup>a</sup>
Aneuploid	Aneuploid	212
Aneuploid	Polyploidy	3
Aneuploid	Mosaic detrimental	47
Aneuploid	Mosaic benign	10 <sup>a</sup>
Aneuploid	Normal	21 <sup>a</sup>
Haploid	Haploid	5
Haploid	Normal	3 <sup>a</sup>
Complex abnormal <sup>b</sup>	Mosaic detrimental	125
Complex abnormal <sup>b</sup>	Aneuploid	34
Complex abnormal <sup>b</sup>	Haploid	19
Complex abnormal <sup>b</sup>	Mosaic benign	4 <sup>a</sup>
Complex abnormal <sup>b</sup>	normal	11 <sup>a</sup>
Polyploid	Polyploid	17
Polyploid	Mosaic benign	2 <sup>a</sup>
Total		885
Misdiagnosed (%)		64 (7.2)

<sup>a</sup>PGD misdiagnosis.<sup>b</sup>Complex abnormal embryos had three or more abnormal chromosomes in the cell biopsied for PGD not consistent with haploidy or polyploidy.

differences in the maternal age between the main groupings ( $P < 0.001$ ) (Table 6). The main deviations were due to the mitotic aneuploid group having a higher average age than the remaining groups.

The average maternal age of the mitotic aneuploid group was also compared with that of normal embryos, and again was shown to be significantly higher ( $P < 0.001$ ). Similar differences between mitotic aneuploid mosaics and normal embryos were observed for both discarded ( $P < 0.05$ ) and PGD embryos ( $P < 0.01$ ). All these findings were obtained from an analysis of variance carried out on the maternal ages.

Although the differences for paternal age were on the borderline of statistical significance, this was almost certainly due to association between the maternal age and the paternal age (Table 7). The data were subjected to a discriminant analysis, which seeks the best linear function of the variables to discriminate between the groups. The weighting coefficients, which reflect the relative importance of the variables, were 2.00 for the maternal age and 0.28 for the paternal age. Thus the paternal age apparently added little to the discriminating power provided by the maternal age.

Per age group (30–34.9, 35–39.9, and 40–45 years), there were 184, 203 and 169 mosaics respectively. For those, the rate of chaotic mosaics did not change (50, 46.3 and 47.3%

respectively), nor did that for 2N/pol (29.9, 27.1 and 20.1% respectively), but there was an obvious increase with maternal age in the mitotic aneuploid group (17.4, 25.6 and 31.9% respectively). The difference between 30–34.9 and 40–45 age groups was statistically significant ( $P < 0.005$ ).

## Risk of PGD misdiagnosis

To determine the error rate of PGD, the embryos not replaced after PGD had their remaining cells disaggregated and analysed with the same protocols used for PGD. This included some chromosomally normal ones with poor development or that did not reach blastocyst stage.

Two types of misdiagnosis produced by mosaicism can occur during PGD. The first is to misdiagnose a detrimental mosaic as normal. This occurred in 4.3% of diagnoses, and was mostly produced by chaotic and mitotic aneuploid mosaics. The second error is to misdiagnose a benign mosaic as abnormal, which occurred in 1.3% of diagnoses and was mostly produced by erroneous classification of a 2N/Pol benign mosaic as normal. In total, the PGD error rate produced by mosaicism is 5.6% (Table 8).

Of the 927 embryos analysed by PGD, 42 (4.5%) did not produce a result after PGD, either because the cell did not have a nucleus or the fixed cell was not informative. Of the 885 embryos with PGD results, 64 (7.2%) were misdiagnosed (Table 9).

## Discussion

The data from patients analysed here are not truly representative of the general population because these patients are infertile, and therefore probably (i) more prone to chromosome abnormalities in their embryos, at least at the cleavage stage, and (ii) because the embryos have been produced by hormonal stimulation to induce superovulation. In the past it has been shown that some types of stimulation produce more mosaic embryos than others (Munné *et al.*, 1997). In addition, about half of the embryos tested here were unsuitable for transfer because of suboptimal morphology and development, which has previously been related to increased chromosome mosaicism (Munné *et al.*, 1995; Márquez *et al.*, 2000). The other half of the embryos analysed by PGD was mostly for the indication of advanced maternal age. Therefore, the mean maternal age of the embryos analysed is substantially higher than the one found in regular IVF patients and in the general population. Nevertheless, from this population of embryos it has been possible to observe interesting phenomena that may reasonably be extrapolated to other embryo groups.

## Onset of mosaicism and formation mechanisms

The onset of mosaicism in cleavage-stage embryos can be determined by assessing the number of blastomeres of each cell line, assuming that by day three of development, no cell death has yet occurred. This can only be accomplished when the majority of cells in a given embryo are analysed. All blastomeres of monospermic embryos are abnormal when the chromosome abnormality occurs during the first embryonic division. When 25 or 50% of the blastomeres are abnormal,

mosaicism arises at the second and third division respectively (Munné *et al.*, 1994a). In the same study, it was also reported that the onset of mosaicism in polyploid and haploid mosaic embryos is usually at the first division, whereas monospermic diploid mosaics were usually generated at the second or later divisions. However, that study was performed analysing only two chromosome pairs. With up to eight chromosome pairs analysed per cell, more mosaics can be detected. The present data show that 51% of diploid mosaics occur at the first mitotic division compared with polyploid mosaics (73%) and haploid mosaics (80%) (Munné and Cohen, 1998). However, the type of diploid mosaicism is also important. Chaotic mosaics are mostly generated during the first mitotic division (73.3%) compared with only 15.3% of 2N/pol, which are mostly produced during the third division (51.4%).

Dispermic embryos and polyploidy or haploid mosaics have similar rates of first mitotic mosaicism as chaotic mosaics, and they are produced by an abnormal number of male centrioles (haploids none, polyspermics two), or suboptimal centriole function (Palermo *et al.*, 1994). In both cases, the first mitotic spindle will not form properly, creating two different chromosomally abnormal cells. Chaotic mosaics may also have a similar mechanism of formation and be predominantly caused by a male factor (the centriole). Abnormal centrioles may be the explanation for male-originated IVF failure in some patients. We have recently identified a couple that produced only mosaic embryos, mostly chaotic, when autologous spermatozoa were used but not when the spermatozoa were donated (Obasaju *et al.*, 1999). Similarly, embryos produced from testicular biopsy in cases of non-obstructive azoospermia have a high incidence of chaotic mosaicism (Silber *et al.*, unpublished data). However, not all chaotic mosaics may necessarily be the result of an abnormal sperm centriole. Instead, they may also be produced by over-expression of factors regulating the centrioles. For instance, human cells that over-express a serine/threonine kinase named STK15 (and not necessarily a male factor) associated with centrosomes (Zhou *et al.*, 1998) have an amplified number of centrosomes, thus miss-segregating chromosomes and producing mosaic tissues (Doxsey *et al.*, 1998). In addition, abnormal centrioles may be a feature of testicular sperm extraction embryos but not necessarily from embryos obtained by plain intracytoplasmic sperm injection (ICSI) because when embryos from ICSI and regular insemination have been compared, no differences were found regarding chromosome abnormalities (Munné *et al.*, 1998c).

It appears that embryos with only a fraction of their cells abnormal, such as most 2N/pol and more than half of aneuploid mitotic mosaics, are caused by culture conditions rather than abnormal gametes. For instance, a drop in temperature may affect cytokinesis, resulting in diploid/polyploid embryos or embryos with multinucleated blastomeres. Spindle microtubules are highly thermosensitive and even a small change in temperature can disturb the spindle structure, which may cause mitotic non-disjunction. It has already been found that embryos from different laboratories cultured under different conditions and stimulation protocols have very diverse rates of mosaicism (Munné *et al.*, 1997).

## Implications for PGD

Up to 4.4% of detrimental mosaics were misdiagnosed by PGD as normal, while 1.3% of benign mosaics were misdiagnosed as abnormal. In total, the PGD error rate produced by mosaicism was 5.7%. When comparing this figure with 7.2% of misdiagnosis after PGD (this study), a sizeable portion of PGD errors was produced by mosaicism. Other sources of errors are signal overlap, which increases with decreasing nuclear diameter (Munné *et al.*, 1996), and split signals, mostly observed with centromeric probes (Munné *et al.*, 1998b). In addition to this 7.2% error rate, embryos classified by PGD as being of a certain kind of abnormality (aneuploid, haploid, polyploidy, etc.) may be reclassified after full embryo reanalysis as another type of abnormality, also because of mosaicism, but generally this type of error is irrelevant to the fate of the embryo and the PGD diagnosis for the patient.

## Maternal age effect

By analysing a large series of mosaic embryos, a clear increase in mitotic aneuploid mosaicism with maternal age was detected, something not previously detected with smaller series (Munné *et al.*, 1995; Munné and Cohen, 1998; Márquez *et al.*, 2000).

These embryos were most probably generated by mitotic non-disjunction. The alternative is that they were produced by a meiotic error, and later some cells became disomic through a mitotic error. Two mechanisms are proposed. In the first instance, the products of the mitotic error were present in the embryo, since both monosomic and chaotic cells may survive up to day 4 of development (Sandalinas *et al.*, 2001). In that case, the embryo would have been classified as being aneuploid with mitotic aneuploid mosaicism. This was observed in some embryos. The second mechanism proposes that disomic cells are a product of the mitotic error and survive better to day 3 than other aneuploid cells, thus suggesting a diagnosis of mitotic aneuploid mosaicism instead of meiotic aneuploidy. This possibility is unlikely because embryonic genome activation is by no means complete until late day 2 or early day 3 (Braude *et al.*, 1988; Tesarik *et al.*, 1988), and thus there is no mechanism to select against chromosomally abnormal embryos until later stages. In addition, this mechanism would imply that such embryos would have a significantly lower cell number than that of their cohort, which has not been observed.

Therefore the evidence suggests that the embryos here classified as mitotic aneuploid mosaics are mostly produced through post-zygotic errors. Therefore their increase with maternal age is in contradiction to commonly held notions that aneuploidy of mitotic origin is maternal age-independent. This assumption is mostly based on data from studies on trisomy 15, 18, and 21 (Antonarakis *et al.*, 1993; Sherman *et al.*, 1994; Eggermann *et al.*, 1996; Lamb *et al.*, 1996; Robinson *et al.*, 1996). However, a recent study has suggested that there may be an increase of post-zygotic maternally linked trisomy X with maternal age (Thomas *et al.*, 1991). It is probable that the mosaics described after IVF are not the ones producing full trisomic conceptions because most have diploid cell lines, which should survive at least as well as trisomic ones.

Maternal age has generally been linked to an increase in meiotic and not mitotic errors. The explanation for the results in this study may depend on the unique events of first embryonic divisions (the cleavage stage). The earliest cleavage divisions in mammals are different from subsequent ones in that they depend mostly on stored maternal mRNA and other oocyte components (Flach *et al.*, 1982; Braude *et al.*, 1988; Tesarik *et al.*, 1988). Recently, Bean *et al.* (2001) have analysed the embryos of a strain of mice (BALB/cWt) that is prone to mitotic non-disjunction of chromosome Y, and found that such mosaicism occurs mostly in the first and second mitotic division. We propose that because the cellular apparatus involved in meiosis is very similar to the one involved in mitosis (Moore and Orr-Weaver, 1998; Toht *et al.*, 2000; Van Heemst and Heyting, 2000), its damage or malfunctioning may result in either meiotic errors or mitotic errors occurring during the cleavage stage when the mRNA and protein pools are still the same as those found in the oocyte. Our findings, if confirmed, may also indicate that the cellular components common to mitosis and meiosis may be as affected by maternal ageing as those specific to meiosis.

In conclusion, the explanation for high rates of mosaicism found in cleavage-stage IVF embryos might be found in (i) genetic factors, such as the Y chromosome in BALB/cWt (Bean *et al.*, 2001); (ii) environmental factors such as temperature and hormonal regimes (Munné *et al.*, 1997); or (iii) the expression of oocyte factors affected by maternal age (present results). Any and all of these factors could disturb this sensitive developmental stage causing a high rate of mosaicism. High rates of mosaicism may also reflect an early selection against chromosome abnormalities (Sandalinas *et al.*, 2001).

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