

Article

Differences in chromosome susceptibility to aneuploidy and survival to first trimester



Dr Santiago Munné

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 250 PGD cycles for translocations and over 2100 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 purpose of this study was to find specific rates of aneuploidy in cleavage-stage embryos compared with first trimester data and to evaluate post-zygotic selection against aneuploidy. A total of 2058 embryos were analysed by fluorescence in situ hybridization (FISH), and specific aneuploidy rates were obtained for 14 chromosomes. Data from morphologically abnormal embryos could be pooled with data from preimplantation genetic diagnosis (PGD) cycles because it was observed that they had similar rates of aneuploidy; thus, for the purpose of studying aneuploidy they could be, and were, pooled. Specific chromosome aneuploidy rates were not related to morphology or development of the embryos. The average maternal age of patients with aneuploid embryos was significantly higher than the overall analysed population. Monosomy appeared more commonly than trisomy. The chromosomes most frequently involved in aneuploidy were (in order) 22, 16, 21 and 15. When compared with first trimester pregnancy data, aneuploidies detected at cleavage stage seem to die in excess of 90% before reaching first trimester, with the exception of chromosome 16 and gonosomes (76% and 14% respectively). Differences in chromosome-specific aneuploidy rates at first trimester conceptions are probably produced by different chromosome-specific aneuploidy rates at cleavage stage and different survival rates to first trimester.

Keywords: aneuploidy, chromosome, first trimester, monosomy, preimplantation genetic diagnosis, trisomy

Introduction

The purpose of this study was to determine chromosome-specific rates of aneuploidy in cleavage-stage embryos. This information was then used to determine if post-zygotic selection was partly responsible for the specific chromosome rates of trisomy observed in clinically recognized conceptions. This was achieved by comparing aneuploidy rates for 14 different chromosomes in cleavage stage embryos and first trimester conceptions.

Most of the evidence for different chromosome susceptibilities to aneuploidy has been obtained from clinically recognized

pregnancies. Major studies have reported that trisomy 16 accounts for 20–35% of all trisomies; acrocentrics and chromosome 2 for 5–10% each; and the rest for lesser proportions (Hassold *et al.*, 1984; Warburton *et al.*, 1986). It is generally accepted that trisomy 16 occurs more frequently than other rare, but possibly underestimated, trisomies (Warburton and Kinney, 1996). If the prevalence of some trisomies is not due to ascertainment biases, these trisomies should have a higher survival rate from zygote to clinical recognition and/or be produced in greater numbers. While there is compelling evidence against different survival rates (reviewed by Warburton and Kinney, 1996), others have found the predominant types of aneuploidies in cleavage stage embryos

(Bahçe *et al.*, 1999) and empty sacs (Schmidt-Saroli *et al.*, 1998) to be different from those found in spontaneous abortions: e.g. trisomy 1 has been described in sperm and embryos (Watt *et al.*, 1987; Spriggs *et al.*, 1996; Shi and Martin, 2000) but very rarely in first trimester conceptions (Hanna *et al.*, 1997).

The study of preimplantation embryos, oocytes and gametes could supply direct evidence of chromosome differences in aneuploidy susceptibility. Extensive studies have been performed in human spermatozoa that demonstrate a similar rate of aneuploidy for all chromosomes, with the exception of gonosome and chromosome 21 disomies, which are slightly more common (Spriggs *et al.*, 1996; Shi and Martin, 2000). As most aneuploidies are maternal in origin, the study of oocytes should provide the best information. Extensive studies on human oocytes and embryos have demonstrated the expected increase in aneuploidy with maternal age, but scant data are available on specific chromosomes (Munné *et al.*, 1995; Dailey *et al.*, 1996; Angell, 1997; Márquez *et al.*, 2000; Sandalinas *et al.*, 2002). Furthermore, data from preimplantation studies cannot be fully extrapolated to the general population, for several reasons: the embryos are obtained from infertile couples after the application of exogenous hormones and drugs; the gametes and embryos are maintained in an artificial environment; the majority of embryos studied may be developmentally and morphologically sub-optimal or defective and often not suitable for replacement or cryopreservation; the couples are older than average; and the data obtained using karyotype analysis are questionable (Almeida and Bolton, 1996) and contradict fluorescence in-situ hybridization (FISH) results (Munné *et al.*, 1995).

The present study attempted to solve some of these limitations by: assessing a large number of embryos; including large sets of morphologically abnormal, as well as normal, embryos; stratifying the data by maternal age, source and morphology; and using FISH to analyse most cells of each embryo to avoid underestimation of mosaicism (Almeida and Bolton, 1996).

To obtain chromosome-specific aneuploidy rates that are representative of the full cohort of IVF embryos, data from morphologically abnormal embryos was pooled with data from preimplantation genetic diagnosis (PGD) cycles, with PGD embryos being the only ethically acceptable source of morphologically normal embryos to be analysed. Because most aneuploidy is believed to be generated at maternal meiosis, aneuploidy does not affect embryo development until morula stage (Munné *et al.*, 1995; Márquez *et al.*, 2000), and because it was observed in this study that the two groups of embryos used had similar rates of aneuploidy, for the purpose of studying aneuploidy they could be, and were, pooled. All these steps should substantially mitigate any ascertainment bias.

The findings indicate different susceptibilities to aneuploidy for different chromosomes, but also varying mortality rates from zygote to first trimester.

Materials and methods

Source of human embryos

All embryos were obtained from patients undergoing IVF at The Institute for Reproductive Medicine and Science at Saint Barnabas Medical Centre (Livingston, NJ, USA). The patient data were investigated in accordance with guidelines approved by the Institutional Review Board (IRB). Separate consents were obtained for embryo research and PGD in accordance with IRB guidelines.

Patients excluded from the study were those with a known altered karyotype, such patients presenting for PGD of translocations, sex chromosome abnormalities or inversions. However, patients with previous trisomic conceptions were included in the study, because the literature indicates that germ line mosaicism is a minor factor in such events, while maternal age is the major contributor.

Two groups of embryos were distinguished according to their source: Group 1 embryos were donated for research because they had compromised morphology and/or development and were supernumerary to those embryos selected for embryo replacement or cryopreservation. Severely compromised development included embryos with fewer than four cells on day 3 (day 0 is retrieval day), or with a normal number of cells but excessive fragmentation or multinucleation on day 2 of development (Alikani *et al.*, 1999, 2002). Group 2 embryos were those analysed after PGD. Group 2 embryonic development ranged from severely compromised to normal, and included embryos that without PGD could be considered suitable for transfer or cryopreservation.

The above embryos were also divided into two groups according to development. Group A were severely compromised embryos, as defined above, and included embryos from groups 1 and 2, and Group B embryos had normal development and were all from Group 2.

As those in Group 1 were spare embryos, they could be disaggregated on day 3 or 4 of development, and all or most of their blastomeres were fixed as described previously (Munné *et al.*, 1998b). Only those embryos with two or more cells with FISH results were included. Group 2 embryos were obtained from patients undergoing PGD. The population of PGD patients is not representative of the whole IVF population, because it consists of patients with advanced maternal age, previous miscarriages, previous trisomic conceptions, repeated IVF failure or a combination of these four factors. Nevertheless, embryos from Group 2 were included in the study as the only sizeable source of morphologically normal embryos currently available for analysis. A fraction of PGD embryos was also morphologically and developmentally compromised.

Embryo analysis

The PGD embryos (Group 2) had one cell biopsied on day 3 of development (Grifo, 1992). Those embryos considered to be chromosomally, morphologically and developmentally normal were replaced. Many of the embryos classified as normal by PGD were transferred to the patient on the same day as the

analysis. The non-transferred embryos, either chromosomally normal or abnormal, were re-analysed with all or most of their cells fixed individually as described previously (Munné *et al.*, 1998b). Infrequently, some chromosomally and morphologically normal embryos, in excess of those transferred, were disaggregated and analysed, because survival after cryopreservation of biopsied embryos is inadequate (Magli *et al.*, 1999). Group 2 embryos included in this study were selected from only those PGD cycles in which all or most of the supernumerary embryos (not replaced) were re-analysed and had at least two cells yielding FISH results. The exceptions were replaced by chromosomally normal embryos that had had only one cell analysed. They were included in this study to provide a proper denominator for calculating aneuploidy rates, even though they could not be re-analysed. This inclusion criterion may represent a minor underestimation of chromosome abnormalities based on prior estimates of false negative FISH error rates (4.1%, as determined below).

Fixed cells were simultaneously analysed with at least four chromosome-specific probes for chromosomes X, Y, 1, 4, 6, 7, 13, 14, 15, 16, 17, 18, 21 and/or 22. Because the embryos included in this study were obtained over some years, the chromosomes analysed also changed with time. The protocols used were previously described by: Munné and Weier (1996) (X, Y, 13, 18, 21); Munné *et al.* (1998a) (X, Y, 13, 16, 18, 21); Munné *et al.* (1998b) (X, Y, 13, 14, 15, 16, 18, 21, 22); Bahçe *et al.* (1999) (1, 4, 6, 7, 14, 15, 17, 18, 22); and Bahçe *et al.* (2000) (X, Y, 1, 13, 15, 16, 18, 21, 22). Data from about half the embryos included here have been described in previous studies (Munné *et al.*, 1998b; Bahçe *et al.*, 1999, 2000; Márquez *et al.*, 2000). All probe solutions included the probe for chromosome 18, which worked as an internal control between protocols. The same probes for each chromosome were used for all the different studies. These probes were X (alpha satellite, DXZ1; Vysis, Downers Grove, IL, USA), Y (satellite III, DYZ1; Vysis), 1 (alpha satellite, D1Z5; Vysis), 4 (alpha satellite, D4Z1; Vysis), 6 (alpha satellite, D6Z1; Vysis), 7 (alpha satellite, D7Z1; Vysis), 13 (RB-1 locus, 13q14, spanning 440 kb; Vysis), 14 (region 14q11.2; developed by Ulli Weier at Lawrence Berkley National Laboratories), 15 (alpha satellite, D15Z4; Vysis), 16 (satellite II, D16Z3; Vysis), 17 (alpha satellite, D17Z1; Vysis), 18 (alpha satellite, D18Z1; Vysis), 21 (region 21q22.13–q22.2; Vysis) and 22 (region 22q13.3; Vysis). All these probes are commercially available, with the exception of the one for chromosome 14.

These probes were used as sequential multiplex sets. Sets of probes always used as the first panel contained probes for chromosomes 13, 16, 18, 21 and 22, or for chromosomes X, Y, 13, 18 and 21. These two probe sets are commercially available (Vysis) and have been properly tested by the provider (Tepperberg *et al.*, 2001). Also always used as first panel was a multiplex probe set prepared by the present authors' laboratory for chromosomes X, Y, 13, 16, 18 and 21 (Munné *et al.*, 1998a), and another one for chromosomes 4, 7, 15, 18 and 22 (Bahçe *et al.*, 1999).

Probe sets used in the second panel were all made in-house and were for chromosomes 14, 15 and 22 (Munné *et al.*, 1998b); for chromosomes 1, 6, 14, 17 and 18 (Bahçe *et al.*, 1999); for chromosomes X, Y, 1 and 15 (Bahçe *et al.*, 2000); for XY, 7,

15 (unpublished); and for XY, 15, 17 (unpublished). Multiplex sets mixed by the present authors' laboratory have been previously tested according to the standards and guidelines for Clinical laboratories of The American College of Medical Genetics (1999). Briefly, 20 interphase chromosomally normal lymphocytes (Vysis) were analysed per multiplex probe set in five different individuals, for a total of 100 cells and 200 distinct genomic targets. Cells classified as errors were those in which one or more of the chromosomes analysed had a count different from two. The error rate for each multiplex probe set and per round of FISH ranged from 1–2% per set (not per chromosome). They were all tested in first hybridizations and not sequentially.

The error rate for sequential experiments was not calculated in lymphocytes, but a 2–4% error rate would be expected, as a result of the combination of the errors in the first and second hybridizations. Because DNA can be damaged through sequential hybridizations, a probe set used in a second hybridization may yield a slightly higher error than if used in a first hybridization. However, a recent study indicates that the error rate per probe set is related to the probe type more than the sequential order of the hybridization (Abdelhadi *et al.*, 2003). Indeed, the rate of false negatives (embryos classified by PGD as normal and found abnormal after reanalysis) is 4.1% (2/49 embryos) when the data of previous reports are added to that of embryos included in this study (Munné and Weier, 1996; Munné *et al.*, 1998a; Munné *et al.*, 1998b; Bahçe *et al.*, 2000).

In order to rule out monosomic diagnoses produced by false negative errors, samples originally analysed after December 2000 were re-analysed using a subtelomeric probe for the chromosome supposedly monosomic, following protocols previously published for the analysis of translocations (Munné *et al.*, 2000). All subtelomeric probes used bound to different loci from those previously analysed to minimize error.

Data evaluation and scoring criteria

For the study of aneuploidy, data from embryos in groups 1 and 2 were combined, following this reasoning: aneuploidy occurs mostly at maternal meiosis I (Hassold *et al.*, 1987, 1988; Antonorakis *et al.*, 1991; Fisher *et al.*, 1995; Eggerman *et al.*, 1996) and mitotically generated aneuploid embryos can still be recognized as mosaics on days 3 and 4 of development by analysing all or most cells of an embryo (Munné *et al.*, 1994b). Even embryos carrying lethal aneuploidies, such as most monosomies, often arrest shortly after genome activation (Sandalinis *et al.*, 2001) but are still available for analysis because arrested cells usually remain alive at least up to developmental day 4.

Exceptions were made for chromosomally normal embryos replaced after analysis of only one cell. These were included to estimate the number of normal embryos and a small proportion (false negative error rate is 4.1%, as noted in the materials and methods section) may in fact be mosaics although rarely aneuploid.

Another reason to combine groups 1 and 2 for the aneuploidy calculation is that previous observations showed that, unlike other chromosome abnormalities, aneuploidy is as often found

in developmentally compromised embryos as in normally developing ones (Munné *et al.*, 1995; Márquez *et al.*, 2000). Karyotype studies reported more aneuploidy in abnormally developing embryos, but data was based mostly on one cell per embryo, which can overestimate aneuploidy and underestimate mosaicism (Almeida and Bolton, 1996). In fact, in embryos derived from euploid oocytes only mosaicism and polyploidy was found to increase with decreasing developmental competence (Munné *et al.*, 1995; Márquez *et al.*, 2000). This is because the types of mosaics found in cleavage-stage embryos are mostly (>70%) caused by chaotic divisions and polyploidization; only a small fraction is caused by mitotic nondisjunction (Munné *et al.*, 2002). The pooling of groups 1 and 2 was also based on the present findings that there were no major differences in individual aneuploidy rates between morphologically normal and abnormal embryos (**Table 1**), because aneuploidy of meiotic origin does not impact embryo morphology or development until genome activation (4–8-cell stage) (Braude *et al.*, 1988; Tesarik *et al.*, 1988). However, morphological abnormalities such as an impaired centrosome and/or multinucleation will cause chromosome mosaicism of the types usually found in dysmorphic embryos (chaotic mosaics, euploid/polyploid mosaics) (Palermo *et al.*, 1994; Zhou *et al.*, 1998).

Classification of chromosomal abnormalities in cleavage-stage embryos, usually with two to 12 cells, requires scoring criteria based on the analysis of as many cells as possible to differentiate mosaicism (30% of cleavage-stage embryos) (Munné *et al.*, 1995) from FISH errors (10% of single cells analysed) (Munné *et al.*, 1998b). In this study, the previously described criteria for distinguishing mosaics from FISH errors were used without modification (Munné *et al.*, 1994a) but only embryos with 3/8 or more abnormal cells were counted as mosaics. When fewer than eight cells were analysed the same cut-off was used but in percentage value, i.e. 37.5%.

We also used previous criteria for single-cell analysis to differentiate signals from two homologue chromosomes in close proximity from the signal of a stretched single chromosome, which could be mistaken for two signals (Munné and Weier, 1996). Embryos were classified as normal, aneuploid, polyploid, haploid and/or mosaic according to guidelines described elsewhere (Munné *et al.*, 1994a, 1995; Harper *et al.*, 1995; Harper and Delhanty, 1996; Munné and Cohen, 1998).

In order to include the whole cohort of embryos in PGD cycles, those embryos classified as normal based on a single cell analysed, and later replaced, were also included in the study. These embryos could still be mosaics, and mosaicism rates may be being underestimated by as much as 5.6%, according to the present authors' most recent study on mosaicism (Munné *et al.*, 2002). Nevertheless, it was considered that not including single-cell analysed normal embryos would overestimate aneuploidy rates, which are the goal of the study.

Statistics

The analyses need to account for a hierarchical structure in order to avoid a nested system of errors, because a varying number of aneuploidy tests were carried out on varying numbers of embryos from 488 patients. Initially, all the analyses were carried out on the highest stratum, i.e. between patients; this was

relaxed only if there was no evidence of heterogeneity of errors. Direct comparisons between group proportions, typically the incidence of aneuploidy, were made using Fisher's exact test. More elaborate analyses, involving several classes of proportions, and incorporating variables such as age, employed the technique of logistic regression, using the algorithm GENSTAT. In order to investigate departures from 1:1 ratios in individual cases, the tail probabilities of the binomial distribution, with a parameter value of 0.5, were used to make inferences. For longer sequences, a sign test was used, in which the number of differences in the same direction was used as a test statistic. Although logistic regression does the analysis on a transform of the proportions, for ease of interpretation all the summary statistics in the tables represent back-transformations onto the original scale of proportions. **Table 1** displays specific aneuploidy rates for each chromosome, in all the groups. Incidences have been adjusted for maternal age by fitting a linear model, where the dependent variable was the logistic transform of the proportions in question, and the independent (explanatory) variables were the patient group (1, 2 and A, B) and the age groupings (up to 35, 35–39, 40 and older). The fitted model was then able to provide estimates of the incidence in one classification (age group). Although the analyses were conducted on the logistic scale, for ease of interpretation, the estimates have again been back-transformed onto the scale of proportions.

Results

In total, 319 embryos had four chromosomes analysed (XY, 13, 18, 21); 580 had five (mostly XY, 13, 16, 18, 21); 84 had six (mostly XY, 13, 16, 18, 21, 22); 750 had eight (XY, 1, 13, 15, 16, 18, 21, 22 or XY, 7, 13, 15, 16, 18, 21, 22 or XY, 13, 14, 15, 16, 18, 21, 22 or XY, 13, 15, 16, 17, 18, 21, 22); 91 had nine (XY, 13, 14, 15, 16, 17, 18, 21, 22); and 234 had 10 (mostly 1, 4, 6, 7, 14, 15, 18, 17, 21, 22 or XY, 1, 4, 7, 13, 15, 16, 18, 21, 22 or XY, 1, 7, 13, 15, 16, 17, 18, 21, 22).

The average number of cells analysed per chromosomally abnormal embryo was 5.1 (range 2–15). The minimum of two cells analysed per embryo only occurred in 11.3% of chromosomally abnormal embryos. Embryos classified as normal by PGD and transferred were also included in the study, and thus only one cell was analysed in 47.2% embryos considered normal, while two cells were analysed in 5.1% of normal replaced embryos, and the remainder had three or more cells analysed because they were not replaced.

Because of the hierarchical structure of the data, the basic experimental unit was initially regarded to be the patient ($n = 488$). It was found that the issue of heterogeneity was negligible and a 'between embryo' analysis could be regarded as reliable ($n = 2058$). A total of 2058 embryos were included in this study, of which 1149 belonged to Group 1 and 909 to Group 2, or 1620 to Group A and 438 to Group B.

Table 1 shows the specific aneuploidy rates for each chromosome in all the groups. All chromosomes had similar rates of aneuploidy in each group of embryos, with the exception of chromosomes 18, which had slightly a higher aneuploidy rate in Group 2 than in Group 1 ($P < 0.05$) and chromosomes 18 and 21, which had a higher aneuploidy rate in Group B than Group A ($P < 0.01$ and $P < 0.05$, respectively).

Accurate estimates of specific chromosome aneuploidy rates require large populations. Groups 1 and 2 can be pooled, provided that the overall rate of aneuploidy is comparable and that the distribution of aneuploidy types are similar. The results in **Table 1** show that individual chromosome aneuploidy frequencies are similar between groups 1 and 2 and A and B for all chromosomes studied, with the exception of 18 and 21.

Therefore, because there are more similarities than differences between these groups of embryos, the data could be pooled to determine chromosome-specific aneuploidy rates and average maternal age of aneuploid embryos (**Table 2**).

There were 375 aneuploid embryos with 485 aneuploidy events when double or triple aneuploidies were counted twice

Table 1. Embryo proportions and counts with specific chromosome aneuploidy frequencies by embryo source. The estimated incidences have been adjusted for differential maternal age in the four groups.

Chromosome	Group 1 (discarded)	Group 2 (PGD)	Group A (morphologically abnormal)	Group B (morphologically normal)
XY	0.015 ± 0.004 (843)	0.007 ± 0.003 (898)	0.013 ± 0.003 (1310)	0.007 ± 0.004 (431)
1	0.037 ± 0.011 (324)	0.008 ± 0.005 (235)	0.029 ± 0.008 (411)	0.014 ± 0.010 (148)
4	0.025 ± 0.011 (214)	0.013 ± 0.010 (113)	0.026 ± 0.010 (271)	No data
6	0.015 ± 0.012 (194)	No data	0.015 ± 0.011 (194)	No data
7	0.031 ± 0.012 (207)	No data	0.030 ± 0.011 (229)	No data
13	0.028 ± 0.005 (921)	0.026 ± 0.005 (880)	0.026 ± 0.004 (1381)	0.031 ± 0.008 (420)
14	0.004 ± 0.004 (221)	No data	0.007 ± 0.005 (261)	No data
15	0.044 ± 0.010 (458)	0.041 ± 0.008 (608)	0.043 ± 0.007 (761)	0.044 ± 0.011 (305)
16	0.043 ± 0.007 (778)	0.052 ± 0.007 (887)	0.053 ± 0.006 (1243)	0.041 ± 0.009 (422)
17	0.024 ± 0.009 (310)	0.024 ± 0.008 (299)	0.022 ± 0.007 (470)	0.032 ± 0.013 (139)
18	0.015 ± 0.004 (1149) ^a	0.032 ± 0.006 (909) ^a	0.017 ± 0.003 (1620) ^c	0.041 ± 0.010 (438) ^c
21	0.034 ± 0.005 (1120)	0.049 ± 0.007 (891)	0.038 ± 0.005 (1586) ^b	0.060 ± 0.010 (425) ^b
22	0.069 ± 0.010 (639)	0.055 ± 0.009 (635)	0.067 ± 0.008 (957)	0.053 ± 0.012 (317)

PGD = preimplantation genetic diagnosis.

Number of embryos analysed per chromosome is given in parentheses.

Double aneuploidies are counted twice, once for each chromosome.

Tetrasomies and nullisomies are counted as two trisomies and two monosomies, respectively.

^{a,b} $P < 0.05$; ^c $P < 0.01$.

Table 2. Pooled data on the proportions of embryos with specific aneuploidies.

Chromosome	Analysed embryos (n)	Aneuploid embryos ^a		Total (n)	Total (%)
		Monosomic (n)	Trisomic (n)		
XY	1741	8	13	21	1.2
1	559	8	6	14	2.5
4	327	4	3	7	2.1
6	194	2	1	3	1.5
7	244	4	3	7	2.9
13	1801	35 ^b	18 ^c	53	2.9
14	280	1	2	3	1.1
15	1066	31	19	50	4.7
16	1665	49	37	86	5.2
17	609	9	7	16	2.6
18	2058	23	24	47	2.3
21	2011	56	38	94	4.7
22	1274	50	34	84	6.6
Total	2058	280 ^d	205 ^e	485	
Acrocentrics (13, 14, 15, 21, 22)	–	173 ^f	111 ^g	284	
Non-acrocentrics (others)	–	107	94	201	

Values in parentheses are percentages.

^aDouble aneuploidies counted twice, once for each chromosome; tetrasomies and nullisomies were counted as two trisomies and two monosomies, respectively.

Significance: b versus c = $P < 0.05$; d versus e, f versus g = $P < 0.001$.

or three times (**Table 2**). There were 93 instances of double or triple aneuploid embryos: 26 in Group 2B; 23 in Group 2A; and 44 in Group 1A. There were significant differences ($P < 0.001$) between the numbers of monosomies and trisomies: 280 and 205, respectively. These differences were mostly due to acrocentric chromosomes, which had 173 monosomies and 111 trisomies ($P < 0.001$). **Table 2** gives the distribution of monosomies and trisomies for the 13 chromosomes. Of these, chromosome 13 was the only one displaying a significantly higher number of monosomies than trisomies (binomial test, $P < 0.05$). To ascertain that excess monosomies were not caused by false negative results, all samples fixed after December 2000 were re-analysed with a subtelomeric probe for the chromosome previously diagnosed as monosomic ($n = 54$). Of these, three were reclassified as normal, indicating a 5.5% false negative rate, which does not substantially change the observation of an excess of monosomies. Taking into account this 5.5% error rate, there would be 265 monosomies out of 485 aneuploid events, which is still a significant excess of monosomies ($P < 0.005$).

The chromosomes most frequently involved in aneuploidy were 22 (6.6% of embryos were aneuploid for this chromosome), 16 (5.2%), 21 (4.7%) and 15 (4.7%). The chromosomes least involved in aneuploidy were 14 (1.1%), X and Y (1.2%), and 6 (1.5%). The observation that chromosome 16 was less involved than 22 could be related to the high maternal age of the population. To remove any possible bias, the embryos were stratified in to three maternal age groups (<35, 35–39, >39). The results, shown in **Table 3**, indicate that chromosome 22 is the most susceptible to nondisjunction of all the chromosomes for all maternal age groups. In addition, the increase in aneuploidy with maternal age is not the same for chromosomes 15, 16, 21 and 22, as aneuploidies 16 and 22 have an almost linear increase, while aneuploidies 15 and 21 have a more exponential pattern, as expected from clinically recognized pregnancy data.

The second column of **Table 4** shows the average maternal age

Table 3. Percentage aneuploidy rates per embryo for the most common aneuploidies by maternal age groups.

Chromosome	Maternal age (years)		
	<35	35–39	≥40
1	0.031 ± 0.012	0.028 ± 0.014	0.040 ± 0.021
13	0.018 ± 0.005	0.029 ± 0.007	0.042 ± 0.010 ^a
15	0.022 ± 0.008	0.040 ± 0.011	0.083 ± 0.018 ^c
16	0.020 ± 0.006	0.060 ± 0.010	0.069 ± 0.012 ^c
17	0.009 ± 0.007	0.018 ± 0.009	0.056 ± 0.019 ^b
21	0.022 ± 0.005	0.035 ± 0.007	0.077 ± 0.012 ^c
22	0.031 ± 0.008	0.074 ± 0.013	0.111 ± 0.019 ^c

^a $P = 0.06$; ^b $P < 0.05$; ^c $P < 0.001$.

of the patients without aneuploid embryos. These ages showed no significant differences, ranging from 34.4 to 35.4 years. The third column on **Table 4** shows the average maternal ages of those patients with at least one aneuploid event in one of their embryos analysed for a specific chromosome. When the two columns are compared, the average maternal age of those patients with aneuploidy events in some of their embryos is higher than for those patients without aneuploid embryos for all chromosomes, with the exception of gonosomes and chromosomes 1 and 14, for which aneuploidy did not show a maternal age effect. However, chromosome 14 may still show a maternal age effect with a higher number of embryos analysed, because the difference between patients with and without aneuploid embryos was 2.3 years. It has to be noted that the average maternal age of both groups of patients (with and without aneuploidy) is high compared with other aneuploidy studies in fertile couples, reflecting the higher average age of patients undergoing IVF.

The chromosome-specific aneuploidy frequencies described in **Table 2** are higher than those found in first trimester conceptions (**Table 4**) and indicate a strong selection against all aneuploidies, although the rate of negative selection for gonosomes (75.8%) and chromosome 16 (76.3%) was less severe than for other chromosomes (between 92% and 100) (**Table 4**).

Discussion

Aneuploidy at cleavage stage

Compromised embryo development is associated with mosaicism and polyploidy but not aneuploidy (Munné *et al.*, 1995; Almeida and Bolton, 1996; Laverge *et al.*, 1997; Márquez *et al.*, 2000). At the most, a slightly higher proportion

Table 4. Average maternal age of patients with and without aneuploid embryos.

Chromosome	Average maternal age ± SE	
	Patients without aneuploid embryos	Patients with some aneuploid embryos
XY	35.4 ± 0.25	35.9 ± 1.40
1	34.8 ± 0.47	35.6 ± 1.30
4	35.2 ± 0.62	39.7 ± 1.07 ^a
6	34.4 ± 0.69	41.7 ± 0.32 ^a
7	34.6 ± 0.68	40.2 ± 1.05 ^a
13	35.1 ± 0.25	37.8 ± 0.67 ^b
14	35.0 ± 0.61	37.3 ± 2.36
15	35.2 ± 0.35	37.9 ± 0.81 ^a
16	35.0 ± 0.29	38.2 ± 0.46 ^c
17	35.4 ± 0.45	40.2 ± 0.87 ^c
18	35.1 ± 0.23	37.3 ± 0.75 ^b
21	34.8 ± 0.25	38.2 ± 0.47 ^c
22	34.8 ± 0.33	38.2 ± 0.52 ^c

^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$.

Overall maternal ages in both groups of patients reflect the advanced age of the population studied.

of normally developing embryos are aneuploid compared with slow and arrested embryos (Munné *et al.*, 1995; Márquez *et al.*, 2000). This is not caused by a gene dosage effect, because the monosomy to trisomy ratio in faster embryos is equal to that of slower embryos. It may be that aneuploidies in slower embryos may be obscured by their mosaicism and polyploidy (Munné *et al.*, 1995). Thus, in general, aneuploidy up to the third day of development does not seem to be affected by morphology or developmental potential, and all embryos can be pooled for the purpose of studying aneuploidy.

The findings here indicate that monosomies are significantly more frequent than trisomies ($P < 0.001$), as was also found in two previous studies (Munné *et al.*, 1995; Márquez *et al.*, 2000), with several possible causes. One is misdiagnosis due to overlap of signals or mosaicism, but because most cells of each embryo were analysed (except replaced embryos), it is quite unlikely that all cells had overlaps of signals for the same chromosome. Probe failure is also unlikely, because the most recent samples were re-analysed with subtelomeric probes binding to a different locus, and nearly identical results were found. Mosaicism is unlikely for the same reason, because it would imply that some cells would be monosomic and others trisomic. Thus, when most cells were analysed, very few embryos were diagnosed as aneuploid instead of mosaic. A third possibility is the occurrence of polymorphisms involving the target area, but probes used for chromosomes 13, 14, 21 and 22 were locus-specific targets and therefore non-polymorphic, and the skewed ratio of monosomy/trisomy is evident in these chromosomes as well. In addition, if this mechanism was responsible for a sizeable number of monosomy classifications, the average maternal age of monosomies should be lower than for trisomies.

Nevertheless, the above reasoning is not entirely satisfactory; so it is possible the excess of monosomies may be produced in the oocyte, not through nondisjunction, but through other mechanisms such as anaphase lag, or chromosome detachment from the metaphase plate (Hunt *et al.*, 1995). For example, in one instance in which all the cells and anuclear cellular fragments of an 8-cell embryo were fixed, all the cells were found to be monosomic for chromosome 18, and a chromosome 18 was also found in an anuclear fragment. Perhaps this chromosome was segregated to the cellular fragment during zygote formation, thereby producing a monosomic embryo. Again, perhaps predivision of chromatids (Angell, 1997) results in an even production of monosomies and trisomies, or favours monosomies.

The average maternal age for the aneuploidies studied was higher than that found in the regular population; but this could be caused by the elevated maternal age of patients undergoing IVF. Even so, while the average maternal age was around 35 years for all the embryos analysed, the average maternal age of the aneuploid embryos was significantly higher, ranging between 37 and 42 years. Interestingly, the average maternal age of embryos aneuploid for chromosome 1 and gonosomes, did not increase with maternal age, which is in agreement with first trimester data indicating that some trisomies, and monosomy X, are not related to maternal age (Warburton and Kinney, 1996).

Chromosome differences in aneuploidy rates and negative selection of aneuploidies

Direct evidence of chromosome differences in aneuploidy susceptibility can be obtained from preimplantation embryos. However, as Warburton and Kinney (1996) reviewed, the data from studies on preimplantation human embryos may be unreliable for various reasons (see Introduction). Some of these problems have been solved in this study. For instance, if the data are divided according to maternal age groups and groups of similar ages are compared, the average maternal age of the population studied is found to be irrelevant, showing that morphology is mostly unrelated to aneuploidy. Discrepancies between FISH and karyotyping studies occurred because most karyotyping studies analysed only one cell per embryo, when mosaicism can be evaluated as aneuploidy (Almeida and Bolton, 1996). However, data on aneuploidy rates in clinically recognized pregnancies from infertile and hormonally super-stimulated patients are still lacking. Nevertheless, the information available indicates that pregnancies obtained after infertility treatment result in similar rates of pregnancy loss to those in natural pregnancies (American Society for Reproductive Medicine and Society for Assisted Reproduction Technology, 1998; Wilcox *et al.*, 1999; Pezeshki *et al.*, 2000) and that spontaneous abortions from IVF are equally as likely to be chromosomally abnormal as those from natural cycles (Plachot, 1989).

The present study demonstrates the existence of different chromosome susceptibilities to aneuploidy. Interestingly, the most common aneuploidy was not chromosome 16 but chromosome 22, followed by 16, 21 and 15. Aneuploidies for chromosomes XY, 14 and 6 were the least common. The difference between trisomy 16 and 22 incidences in preimplantation embryos and clinically recognized pregnancies is not caused by the high maternal age of the embryos included in this study (**Table 3**). The increase of trisomy 16 and 21 with maternal age, one linear and the other exponential, are similar to those previously described in clinically recognized conceptions (Hassold *et al.*, 1984; Warburton *et al.*, 1986).

Previous large studies describing individual rates of aneuploidy in cleavage-stage embryos mostly support the present findings, such as the present authors' first large study with 524 embryos analysed for chromosomes XY, 18, 13 and 21 (Munné *et al.*, 1995). In that study, while the 13 and 21 results cannot be directly compared with the present results, the aneuploidy rate for chromosome 18 was double that for gonosomes, as in the present data. The second large study by Márquez *et al.* (2000) analysed chromosomes XY, 13, 16, 18 and 21 in 1255 embryos (data included in the present study) and also found that gonosomes were the least common aneuploidies (1.6%) followed by chromosome 18 (3.1%) and chromosome 21 (5.5%), while in that study chromosome 16 was barely studied in the over-40 population.

Sperm studies show that trisomy 22 is significantly more common than the others (Shi and Martin, 2000), although the 1.2% rate of disomy 22 in sperm cannot account for the high rates of trisomy 22 found in embryos. Spectral karyotyping studies in fresh non-inseminated oocytes also showed that

chromosome 22 had the highest frequency of aneuploidy, followed by the rest of the small chromosomes, with most of the aneuploidy detected being originated by premature predivision of chromatids (Sandalinas *et al.*, 2002). In the same study they argued that oocyte degeneration in older women may imply deficient protein synthesis, as did Steuerwald *et al.* (2001), thus affecting the proteins that maintain cohesion between sister chromatids (see reviews by Cohen and Fix, 2000; Van Heemst and Heyting, 2000). Any disturbance of the equilibrium of these meiosis-specific proteins could result in the premature predivision of chromatids, and the smaller the chromosomes, the more often a suboptimal amount of cohesions may result in extra predivision during meiosis II. For instance, the distal chiasma predisposes nondisjunction of chromosome 21, probably because there is less cohesion distal to the chiasma (Lamb *et al.*, 1997). Even though some techniques used in other oocyte studies did not always differentiate specific aneuploidies, it was also found that group E and G chromosomes had the highest aneuploidy rates, mostly chromatid related (Pellestor *et al.*, 2002).

However, the present data do not correspond well with PGD studies on polar bodies. In a large series of cases (1296 cycles and 6733 eggs analysed), Kuliev *et al.* (2002) found that the chromosomes more involved in aneuploidy were 21 (21%), 22 (18%), 18 (13%), 13 (11%) and 16 (9%) in a population with an average maternal age similar to that in the present study (38.5 years old). The rates of aneuploidy found in those polar bodies were much higher than those found by the present authors in embryos. In addition, there were many more missing chromosomes and chromatids in metaphase I polar bodies than extra chromatids and chromosomes, thus indicating that there should be more disomic eggs and trisomic embryos, i.e., the contrary of what has been found in this study. To determine why the study by Kuliev *et al.* (2002) and the

present study are so different, the analysis of the embryos resulting from oocytes with abnormal polar body results should be analysed.

The present study does confirm previous observations indicating variable lethality frequencies for aneuploid embryos (Schmidt-Saroli *et al.*, 1998; Munné *et al.*, 1998b; Sandalinas *et al.*, 2001). For instance, it was found that 58% of aneuploid events were actually monosomies; whereas, with the rare exception of monosomy 21, autosome monosomies are never detected in spontaneous abortions. Also, when the results were compared with the prevalence of aneuploidy in first trimester conceptions (Simpson, 1990), some trisomies were found to survive to first trimester less frequently than others. For instance, more than 90% of aneuploid embryos are lost between cleavage stage and first trimester (**Table 5**), but for chromosome 16 the loss drops to 76%, and for gonosomes it becomes negligible. If only trisomies are considered, most trisomies are lost at a rate of 80% or higher; but only 45% of trisomy 16 and 14% of trisomy for gonosomes are lost. Aneuploidy 1, which is seldom detected in spontaneous abortions, was found in 2.4% of cleavage-stage embryos. Therefore, even though trisomy 22 is more common than trisomy 16 in cleavage-stage embryos, it seems that trisomy 16 survives to first trimester in higher proportions, and is therefore much more frequently detected than any other trisomy in spontaneous abortions. Monosomy X was actually found to increase in frequency from day 3 of development to first trimester conceptions; but this does not necessarily mean that some monosomy X embryos are generated after day 3. Looking at the proportion of chromosomally normal embryos (about 50% normal; Márquez *et al.*, 2000) to that of first trimester conceptions (90% normal; Simpson, 1990; Thompson *et al.*, 1991), there can be seen nearly a doubling of this proportion. It can therefore be deduced that the ratio of monosomy X to normal embryos does not change much from

Table 5. Estimated percentage embryo loss of aneuploidies from cleavage stage to first trimester.

<i>Chromosome</i>	<i>Aneuploidy in cleavage-stage embryos (%)^a</i>	<i>Trisomy in cleavage-stage embryos (%)^a</i>	<i>Aneuploidy in clinically recognized pregnancies (%)^b</i>	<i>Embryo loss (%)</i>	<i>Trisomy loss (%)</i>
XY tris	0.5	0.5	0.43	14.0	14.0
XY mono	0.8	–	1.47	0	–
1	2.5	1.1	0.00	100	100
4	2.1	0.9	0.11	94.8	88.0
6	1.5	0.5	0.02	98.7	96.1
7	2.9	1.2	0.15	94.8	87.8
13	2.9	1.0	0.19	93.4	81.0
14	1.1	0.7	0.14	96.0	80.3
15	4.7	1.8	0.29	93.8	83.7
16	5.2	2.2	1.23	76.3	44.6
17	2.6	1.2	0.03	98.8	97.4
18	2.3	1.2	0.20	91.3	83.3
21	4.7	1.9	0.48	89.8	74.7
22	6.6	2.7	0.38	94.2	85.8

^aPresent study.

^bFormula: (Aneuploidy in live births, from Thompson *et al.*, 1991) + [(Aneuploidy in spontaneous abortions, from Simpson, 1990) x (17% chance of abortion in women 35 and older, from Schmidt-Saroli *et al.*, 1998)].

day 3 to first trimester, indicating that monosomy X embryos survive from day 3 to first trimester just as well as chromosomally normal embryos. If other aneuploidies are compared with normal embryos, the selection against aneuploid embryos seems even more severe.

It is not surprising that some trisomies survive better to first trimester than others. Different types of chromosome abnormalities are found in empty sacs than those in abortuses that previously had a fetal heartbeat (Schmidt-Saroli *et al.*, 1998). Similarly, some uncommonly detected trisomies (13, 18, 21, gonosomes) in clinically recognized pregnancies can reach birth, while more common ones (16, 22) do not. Nevertheless, the existence of selection against specific trisomies should be substantiated by comparing the present results with data on clinically recognized pregnancies obtained from IVF patients, data that so far are unavailable.

Conclusion

This audit on cleavage-stage aneuploidy demonstrates that more monosomies are produced than trisomies, rather than the 1:1 ratio previously assumed, and that the excess is due to acrocentric chromosomes. However, all but monosomy X and 21 can reach blastocyst stage (Sandalinas *et al.*, 2001).

Of the chromosomes analysed, aneuploidy increased with maternal age, except for gonosomes and chromosomes 1 and 14. That, combined with different patterns of maternal age effect, and different baselines of aneuploidy, explains a great variety of chromosome-specific aneuploidy, unlike sperm data. Interestingly, aneuploidy 22 and not 16 was the most common found in any age group. However, while the mortality rate of most trisomy types from cleavage stage to first trimester was 80% or higher, for trisomy 16 it was only 45%, explaining why trisomy 16 is the most common type found in first-trimester conceptions.

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