

Polar body analysis by array comparative genomic hybridization accurately predicts aneuploidies of maternal meiotic origin in cleavage stage embryos of women of advanced maternal age

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STUDY QUESTION: How accurate is array comparative genomic hybridization (array CGH) analysis of the first polar body (PB1) and second polar body (PB2) in predicting aneuploidies of maternal meiotic origin in the cleavage stage embryos of women of advanced maternal age?

SUMMARY ANSWER: Almost all of the aneuploidies detected in cleavage stage embryos were associated with copy number changes in the polar bodies (93%) and all but one (98.5%) were predicted to be aneuploid. A minority of copy number changes (17%), mainly in PB1, did not result in the predicted changes in the embryo, but many of these were small copy number changes, which are likely to be artefacts.

WHAT IS KNOWN ALREADY: Chromosome aneuploidy is a major cause of pregnancy failure and loss, abnormal pregnancy and live births. Most aneuploidy is of maternal meiotic origin and increases exponentially in the decade preceding the menopause. A pilot study demonstrated a high rate of concordance between the chromosomal status predicted by polar body analysis and the corresponding zygotes in women of advanced maternal age.

STUDY DESIGN, SIZE AND DURATION: Polar body biopsy and array CGH analysis of mature oocytes, which fertilized normally, to identify segregation errors in meiosis, followed by the analysis of the corresponding cleavage stage embryos ($n = 34$), in a consecutive series of stimulated and natural IVF cycles in women of advanced maternal age.

MATERIALS, SETTING AND METHODS: Twenty couples requesting aneuploidy screening (mean \pm SD of maternal age 39 ± 3 years) had 16 controlled ovarian hyperstimulation and 7 natural IVF cycles. PB1 and PB2 were biopsied from mature oocytes, prior to intracytoplasmic sperm injection (ICSI) and following confirmation of normal fertilization, respectively. Array CGH was used to detect chromosome copy number changes and to predict aneuploidy in the corresponding embryos. Embryos with normal copy number in both polar bodies were transferred but, 34 cleavage stage embryos, most of which were predicted to have one or more aneuploidies of maternal meiotic origin, were analysed in whole after removal of the zona by array CGH, on Day 3 post-ICSI.

MAIN RESULTS AND THE ROLE OF CHANCE: Thirty cleavage stage embryos, predicted to have one or more aneuploidies, were all confirmed to be aneuploid (100% concordant). Seventy four aneuploidies were detected in these embryos. Sixty-nine (93%) aneuploidies were associated with copy number changes in the polar bodies and 68 (98.5%) of these had been predicted to be aneuploid. Also, 19 of 20 (95%) balanced combinations of chromatid gain/loss in PB1/PB2 accurately predicted normal copy number in the corresponding embryos. However, 17 (12%) copy number changes in the polar bodies did not result in the expected outcome, including 12 false positive predictions of aneuploidy. Most of these involved copy number changes that were smaller than would be expected for whole chromosome or chromatid imbalance and

occurred significantly more often in PB1 than PB2 ($P < 0.0005$). Three other embryos with only small copy number changes and one embryo with a partial chromosome loss in PB2, were all confirmed to be euploid.

LIMITATIONS, REASONS FOR CAUTION: Accurate false positive and negative rates will require follow-up of both euploid and aneuploid embryos, ideally using molecular genetic markers to detect aneuploidy independently and to identify their origin.

WIDER IMPLICATIONS OF THE FINDINGS: Polar body biopsy and array CGH analysis is efficient and accurately predicts most aneuploidies in cleavage stage embryos. However, the size of the ratio shifts, particularly in PB1, should always be compared with the X chromosome shift before it can be concluded that there is a real copy number change.

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Key words: chromosome aneuploidy / meiosis / pre-implantation genetic screening / polar body / array CGH

Introduction

Chromosome aneuploidy is a major cause of pregnancy failure and loss, abnormal pregnancy and live births following both natural conception and IVF (Hassold and Hunt, 2001; Spandorfer *et al.*, 2004). Most aneuploidy is of maternal meiotic origin and its incidence increases exponentially in the decade preceding the menopause. For this reason, pre-implantation genetic screening (PGS) of human embryos following IVF, typically by either polar body or cleavage stage embryo biopsy and fluorescence *in situ* hybridization (FISH) of five to nine chromosome specific probes to the single metaphase or interphase nuclei, had, until recently, been used extensively in women of advanced maternal age and other indications associated with a higher incidence of aneuploidy (Verlinsky *et al.*, 1996; Munne *et al.*, 2002a). The aim of PGS is to select embryos with the normal number of the chromosomes for transfer and thereby to increase implantation and clinical pregnancy rates, reduce the incidence of miscarriage and avoid abnormal pregnancies and live births. However, numerous randomized controlled trials (RCTs) have failed to demonstrate any improvement in live-birth rates (Harper *et al.*, 2010) and in one large trial of PGS for advanced maternal age, live-birth rates were significantly decreased (Mastenbroek *et al.*, 2007).

The reasons for the lack of improvement in live-birth rates are likely to be a combination of several factors. These include the invasiveness of cleavage stage biopsy that may reduce pregnancy rates (De Vos *et al.*, 2009), the relatively high incidence of chromosome malsegregation errors in cleavage that results in chromosomal mosaicism (Munne *et al.*, 1994, 2002b, 2007; Delhanty *et al.*, 1997) so that a single blastomere may not be representative of the whole embryo, the limited number of chromosomes that can be screened by FISH and the errors inherent in interphase FISH.

It is now possible to use whole genome amplification and microarray based comparative genomic hybridization (array CGH) for accurate copy number analysis of all 24 chromosomes in single blastomeres (Gutierrez-Mateo *et al.*, 2011). Furthermore, pregnancies and live births have been reported following first polar body analysis (Fishel *et al.*, 2010, 2011). Also, a recent RCT has demonstrated that trophectoderm biopsy and transfer of euploid blastocysts later the same day results in a significant improvement in pregnancy and live-birth rates in young women having elective single blastocyst transfers to avoid multiple pregnancies (Yang *et al.*, 2012).

For PGS in women of advanced maternal age, the European Society for Human Reproduction and Embryology (ESHRE) PGS Task Force decided to undertake a pilot study in which both polar bodies were biopsied simultaneously, following intracytoplasmic sperm injection (ICSI), and analysed by array CGH to detect copy number changes and predict aneuploidy in the corresponding embryos (Geraedts *et al.*, 2011; Magli *et al.*, 2011). This approach has the advantages that polar body biopsy is less invasive because the polar bodies do not contribute to the developing embryo, and only errors in female meiosis are detected avoiding any possible diagnostic errors caused by chromosomal mosaicism at later stages of pre-implantation development. This pilot study demonstrated that polar body and array CGH analysis was efficient and could be completed within 12 h. Also, the euploid/aneuploid status of the polar bodies was highly concordant (94%) with the status of the corresponding, mainly aneuploid, zygotes.

Analysis of the pattern of segregation errors of the chromosomes in the two polar bodies and corresponding zygote, demonstrated that almost all errors in the first meiotic division are caused by pre-mature pre-division of sister chromatids and not non-disjunction of whole chromosomes and many oocytes have multiple meiotic errors (Handyside *et al.*, 2012). This is consistent with increasing evidence that meiosis specific cohesins which bind the homologous chromosomes together in oocytes arrested in prophase in the fetal ovary before birth, do not turn over and may be gradually degraded (Handyside, 2012). However, a significant proportion (28%) of chromosome segregation errors detected as copy number changes in the polar bodies did not result in the predicted outcome in the corresponding zygote (Handyside *et al.*, 2012).

To investigate the concordance rate and the accuracy of polar body analysis at the chromosomal level, we report here a detailed retrospective analysis of copy number changes detected by array CGH in the first (PB1) and second polar bodies (PB2) and corresponding cleavage stage embryos, in a consecutive series of controlled ovarian stimulation and natural IVF cycles in women of advanced maternal age with predominantly normospermic partners. To avoid any possibility of misidentification, PB1 and PB2 were biopsied separately, before ICSI and after checking for fertilization the following day, respectively, and where copy number changes were detected in the polar bodies predicting one or more maternal aneuploidies of meiotic origin, the corresponding embryos were followed up on

Day 3 post-ICSI, when most embryos had reached 6–10-cell stages. Array CGH analysis of the whole embryo at these stages has the advantage that any aneuploidy of meiotic origin would normally be replicated in all of the cells and, therefore, should be detected by array CGH with high accuracy. However, if malsegregation of chromosomes does occur during cleavage, any resulting aneuploidy will only be detected if there is a net gain or loss in a majority of cells (Mamas *et al.*, 2012) and the problem of interpreting non-concordant results in single blastomeres or small numbers of trophectoderm cells caused by chromosomal mosaicism is avoided.

Materials and Methods

Patient criteria and ovarian stimulation

Twenty couples requesting PGS for advanced maternal age (≥ 35 years; mean \pm SD of maternal age 39 ± 3 years) combined with a history of repeated implantation failure (≥ 3 cycles; $n = 14$), previous aneuploid pregnancy ($n = 2$) or recurrent first trimester miscarriage ($n = 5$) and one patient carrying a balanced translocation [46,XX,t(16;17)(q24;p11)], underwent 16 controlled ovarian hyperstimulation cycles and 7 natural fresh cycles. The male partners generally had semen parameters in the normal range except for two men, who had oligoasthenoteratozoospermia. Ovarian stimulation was achieved using an antagonist protocol and oocytes were retrieved by ultrasound-guided transvaginal aspiration 36 h after β -human chorionic gonadotrophin (β -hCG) administration. For natural IVF cycles, an ultrasound scan was performed on the second day of the menstrual cycle to exclude the presence of ovarian cysts. Ultrasound monitoring was then performed from the eighth day of the cycle and onwards to measure the size of the follicle and the endometrial thickness. When the average follicular diameter was > 16 mm, 5000 IU β -hCG was administered and oocyte retrieval was performed under local anaesthesia.

Ethical approval

PGS by polar body biopsy and array CGH analysis, and follow-up of embryos, was carried out with the patients' informed consent prior to oocyte collection. The study was approved by the National Authority of Human Reproduction (no. 7/2009), Greece.

Polar body biopsy

Mechanical partial zona dissection and biopsy of the PB1 was performed in microdrops of buffered culture medium under sterile equilibrated tissue culture oil (Sage Inc., Trumbull, CT, USA). PB1 and negative wash drop controls were collected and transferred in minimal medium (less than 0.5 μ l) to 0.2 ml PCR tubes containing 2 μ l phosphate buffered saline (BlueGnome, Cambridge, UK), using separate pulled micropipettes, under a stereo microscope in a sterile hood and stored at -20°C . The micropipette was then flushed to ensure that the polar body had not been retained in the pipette. Following the biopsy, the oocytes were inseminated by ICSI and returned to culture. The following morning, 16–18 h post-ICSI, each oocyte was checked for pronuclei and extrusion of the PB2 to confirm fertilization. PB2 was then biopsied from each normally fertilized oocyte using the existing slit in the zona. PB2 and negative wash drop controls were then transferred to PCR tubes as described.

Follow-up analysis of cleavage stage embryos

Following array CGH analysis of the polar bodies, embryos were selected for follow-up analysis on Day 3 post-ICSI with patients' informed consent. To avoid any residual contamination from cumulus cells, the zona pellucida

was removed from each embryo by micromanipulation using a non-contact laser (Octax Microscience GmbH, Bruckberg, Germany) to dissolve half of the zona which then allowed the embryo to be pulled out of the zona. The zona-free embryo was then washed and placed in a PCR tube as described for the polar bodies.

Whole genome amplification and array CGH analysis

Whole genome amplification (SurePlex; BlueGnome, Cambridge, UK) and array CGH (24sure V2 and 24sure+; BlueGnome, Cambridge, UK) analysis of polar bodies and cleavage stage embryos was carried out according to the manufacturer's instructions. The slides were scanned using a 10- μ m laser scanner (Innopsys 710A; Innopsys S.A., Carbonne, France) and the images analysed (BlueFuse Multi; BlueGnome, Cambridge, UK). The array CGH ratio plots of each polar body were analysed for gains and losses, by at least two independent assessors, and the euploid/aneuploid status of the corresponding embryo predicted. The array CGH ratio plots of the corresponding cleavage stage embryos were also assessed blind by two independent assessors. The results were then collated, and a detailed retrospective analysis of the array CGH plots was undertaken with particular attention to the size of the ratio shifts where copy number changes had been reported. (The chromosomes involved in the translocation were excluded from this analysis.) Theoretically, the array CGH ratio shift for the X chromosome with sex mismatched, male (XY) DNA, known as the X chromosome separation, should be $\log_2(2/1)$ or 1.0. For any autosome with a whole chromosome gain in PB1, the ratio shift should be the same as the X separation, i.e. $\log_2(4/2)$ or 1.0. Whereas, for a chromatid gain, following premature pre-division, the ratio should be $\log_2(3/2)$ or 0.6. Similarly, for whole chromosome or chromatid loss in PB1, the ratios should be > -1.0 and -1.0 , respectively, and for gain or loss of a chromatid in PB2, the ratios should be 1.0 or > -1.0 , respectively.

Statistical analysis

The incidence of small copy number changes in PB1 was compared with PB2 using Fisher's exact test (two tailed) with $P < 0.05$ considered significant.

Results

Polar body biopsy and array CGH analysis

The first polar bodies (PB1) were biopsied from 92 mature oocytes prior to intracytoplasmic sperm injection (ICSI). Of these, 58 (63%) had two pronuclei the following morning, indicating normal fertilization. The PB2 was biopsied from 57 (98%) normally fertilized oocytes, and whole genome amplification and array CGH analysis of both polar bodies were successful in 54 (93%). Copy number changes in the polar bodies predicting aneuploidy of maternal meiotic origin in the corresponding embryo were detected in 39 of 54 (72%) embryos (Fig. 1). In addition, three embryos had copy number changes for one or two chromosomes that were smaller than expected for either whole chromosome or chromatid imbalance (see Materials and Methods), and one embryo had normal copy number for all chromosomes in both polar bodies, except for a partial deletion of the short arm of chromosome 11 in PB2. The remaining 11 (20%) embryos had normal copy number for all chromosomes in both polar bodies.

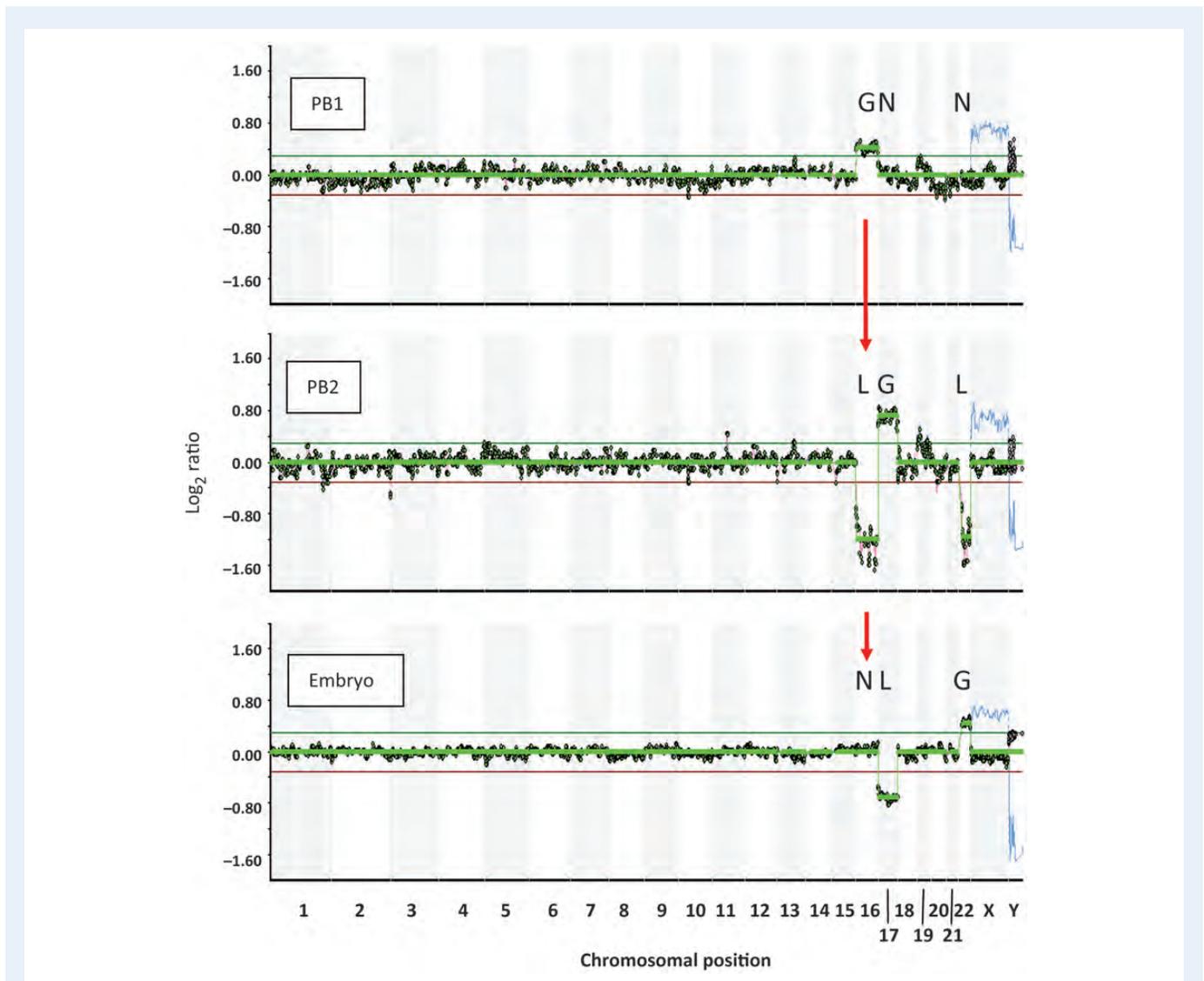


Figure 1 Array CGH ratio plots for the first and second polar bodies (PB1 and PB2) and corresponding 8-cell embryo (Embryo) on Day 3 post-ICSI. There is one error in meiosis I, a chromatid gain (G) for chromosome 16, which is balanced by a chromatid loss (L) in PB2, resulting in normal copy number (N) in the corresponding embryo, as would be expected by pre-mature pre-division of sister chromatids for one of the homologous chromosomes and random segregation of the chromatid in meiosis II. In addition, there are two meiosis II errors, one gain for chromosome 17 and one loss for chromosome 22, both of which resulted in the expected loss and gain in the corresponding embryo. Green and red horizontal lines represent the 95% confidence interval for normal copy number. The blue line visible for the X and Y chromosomes is the ratio shift against the sex mismatched (male) control DNA.

Follow-up of cleavage stage embryos

Thirty cleavage stage embryos predicted to have one or more aneuploidies of maternal meiotic origin, the three embryos with one or two small copy number changes and predicted to have no maternal aneuploidies, and the embryo with the structural abnormality were followed up by array CGH of the whole embryo on Day 3 post-ICSI when most embryos were at 6- to 10-cell stages.

Follow-up array CGH analysis of the 30 embryos predicted to be aneuploid, confirmed that all were aneuploid (100% concordant) with a total of 74 aneuploidies (29 trisomies and 45 monosomies; average 2.5 per embryo, range 1–6) (Table 1). Sixty-nine (93%) of the aneuploidies in the embryos were associated with copy number

changes in the polar bodies and are assumed to be of maternal meiotic origin and the other five (7%), in which both polar bodies had a normal copy number for all chromosomes, to be of paternal or post-meiotic origin. Sixty-eight (98.5%) of the maternal aneuploidies had been predicted to be aneuploid. Overall, copy number changes were detected for 100 chromosomes in one or both polar bodies resulting in the expected gain, loss or normal copy number in 83 chromosomes in these embryos (Table 2). These included 18 segregation errors in the first meiotic division, meiosis I (MI), caused by premature pre-division of sister chromatids, and 45 in the second, meiosis II (MII), that were detected by gain or loss in PB1 or PB2 only, respectively, and resulted in the expected aneuploidy

Table 1 Distribution of maternal meiotic aneuploidies, paternal or post-zygotic aneuploidies and unclassified segregation errors in 30 cleavage stage embryos predicted to be aneuploid following polar body analysis.

Embryo no.	Segregation errors (mainly pre-mature pre-division of sister chromatids)			Post-zygotic or paternal aneuploidies	Total aneuploidies	Unclassified segregation errors	
	Total maternal errors	Net maternal aneuploidies	With small ratio shift			Expected ratio shift	With small ratio shift
1	2	1		1	2	2	
2	1	1			1		
3	1	1			1		
4	1	1			1		
5	5	1			1		
6	1	1			1		
7	3	1			1		2
8	1	1			1		
9	2	1			1	1	
10	1	1			1		
11	1	1		1	2		1
12	3	1		1	2		2
13	5	2			2		
14	2	2			2		
15	3	2			2		
16	4	2	1		2		1
17	3	2	1		2		
18	2	2			2		
19	3	2			2		
20	2	2			2		
21	6	3			3	1	
22	5	3	1		3		2
23	3	3			3		
24	5	3	1	1	4	1	
25	4	4			4		
26	4	4			4		
27	6	5	1	1	6	1	3
28	5	5			5		
29	9	5			5		
30	7	6	1		6		
Total	100	69	6	5	74	6	11

in the corresponding embryo. Only one MI error (5%) was caused by non-disjunction of a whole chromosome as identified by the whole chromosome loss from PB1 and chromatid gain in PB2, and resulted in trisomy in the corresponding embryo. In addition, 19 of 20 (95%) balanced combinations of chromatid gain/loss in PB1/PB2 accurately predicted normal copy number in the corresponding embryos.

The patterns of 17 (17%) copy number changes in the polar bodies and corresponding embryos could not be explained by known types of segregation errors and were, therefore, categorized as unclassified errors (Tables 1 and 2). These included 12 copy number changes in PB1 or PB2 alone that did not result in aneuploidy in the

corresponding embryo (false positive), 4 copy number changes that predicted the opposite aneuploidy from that detected in the corresponding embryo and 1 apparently balanced combination that resulted in trisomy in the corresponding embryo (false negative). Most of these (11 of 17) involved copy number changes that were smaller than would be expected for whole chromosome or chromatid imbalance (Fig. 2). Furthermore, overall these small copy number changes were significantly more frequent in PB1 when compared with PB2 (15 of 52, 29% versus 2 of 70, 3%, $P < 0.0005$).

The three cleavage stage embryos with only small copy number changes, and the embryo with partial loss of the short arm of chromosome 11 in PB2, were all confirmed to be euploid.

Table II Chromosome copy number changes in the polar bodies and the corresponding cleavage stage embryos of 30 embryos predicted to have one or more aneuploidies of maternal meiotic origin.

Copy number		n	Type of segregation error	Expected copy number in the corresponding embryo	No. of chromosomes with expected copy number (%)	No. of unclassified segregation errors (%)	Details (PBI/PB2/embryo)
Polar body 1	Polar body 2						
Gain	Normal	14	MI PD	Loss	9 (64)	5 (36)	GNN, 4x gNN
Loss	Normal	16	MI PD	Gain	9 (56)	7 (44)	2x LNN, 3x INN, INI, LNI
Normal	Gain	31	MII NDJ/PD	Loss	30 (97)	1 (3)	NgN
Normal	Loss	17	MII NDJ/PD	Gain	15 (88)	2 (12)	NLL, NLN
Gain	Loss	12	MI NDJ	Loss	0	1 (8)	gLG
			MI PD balanced in MII	Normal	11		
Loss	Gain	9	MI NDJ	Gain	1	0 (0)	
			MI PD balanced in MII	Normal	8		
Gain	Gain	0				0	
Loss	Loss	1				1	ILG
Total		100			83 (83)	17 (17)	

MI, meiosis I; MII, meiosis II; NDJ, non-disjunction; PD, pre-mature pre-division of sister chromatids; G, gain; L, loss; N, normal copy number; g, small copy number gain; l, loss (array CGH log₂ ratio shift significantly less than expected by comparison with the sex mismatched X chromosome internal control).

Clinical outcome

Eleven embryos (20%) had normal copy number in both polar bodies and were predicted to be unaffected by maternal aneuploidies of meiotic origin. (This included a normal or balanced combination of the translocation chromosomes in the maternal translocation carrier.) One or more unaffected embryos were available for transfer in 9 out of the 23 cycles (39%). Transfer of 10 embryos of good morphology on Day 3 post-ICSI in seven cycles resulted in three clinical pregnancies (12.5% per cycle; 43% per embryo transfer), of which 1 is ongoing and 2 have delivered with one singleton and one twin live births.

Discussion

Polar body biopsy and microarray based array CGH was successful in a high proportion (93%) of normally fertilized embryos and 30 cleavage stage embryos predicted to have one or more aneuploidies of maternal meiotic origin were all confirmed to be aneuploid (100% concordance). These results reinforce the conclusions of the previous pilot study that polar body biopsy, and array CGH is a reliable strategy for clinical application in women of advanced maternal age (Geraedts *et al.*, 2011).

Sixty-nine out of 74 (93%) of the aneuploidies detected in these aneuploid cleavage stage embryos were associated with copy number changes in the polar bodies and 68 (98.5%) of these had been predicted to be aneuploid (Table 1). Thus, women can be reassured that a large majority, if not all, aneuploidies present in cleavage stage embryos can be identified by polar body analysis. However, most of the women in this study had normospermic partners and alternative embryo biopsy strategies may be more appropriate where high

levels of paternal aneuploidy are suspected. Also, although follow-up analysis of the whole cleavage stage embryo provides accurate array CGH results to confirm the presence of aneuploidies of meiotic origin replicated in all cells, aneuploidies caused by malsegregation of chromosomes during cleavage would not be detected unless there is a net gain or loss in a majority of cells (Mamas *et al.*, 2012). Hence, the 7% of aneuploidies not associated with copy number changes in the polar bodies, and assumed to be of paternal or post-meiotic origin, may be an underestimate of aneuploidies with the potential to affect the development of the embryo, which are not detected by polar body analysis.

Of more concern is that 17 of 100 (17%) copy number changes in the polar bodies of these embryos had to be categorized as unclassified segregation errors because they did not predict the outcome in the corresponding embryo accurately, including 12 false positive copy number changes predicting aneuploidy, mainly in PBI (Table 2). Similar results were reported in the previous pilot study in which 78 of 353 (22%) of segregation errors were unclassified, including 73 (21%) false positives, although the incidence in PBI and PB2 was similar (Handyside *et al.*, 2012).

Close inspection of the array CGH plots revealed that many of these unclassified errors involved copy number changes which, by comparison with the ratio for the X chromosome versus sex mismatched control male DNA, were smaller than expected for either whole chromosome or chromatid imbalance (Fig. 2). These small copy number changes are likely to be artefacts of whole genome amplification from single cells and, therefore, should not be reported. However, discriminating these small changes from genuine chromatid imbalances in PBI can be difficult because the array CGH ratio changes are less than those for PB2 (see Materials and Methods). Furthermore, the overall incidence of these small changes was significantly

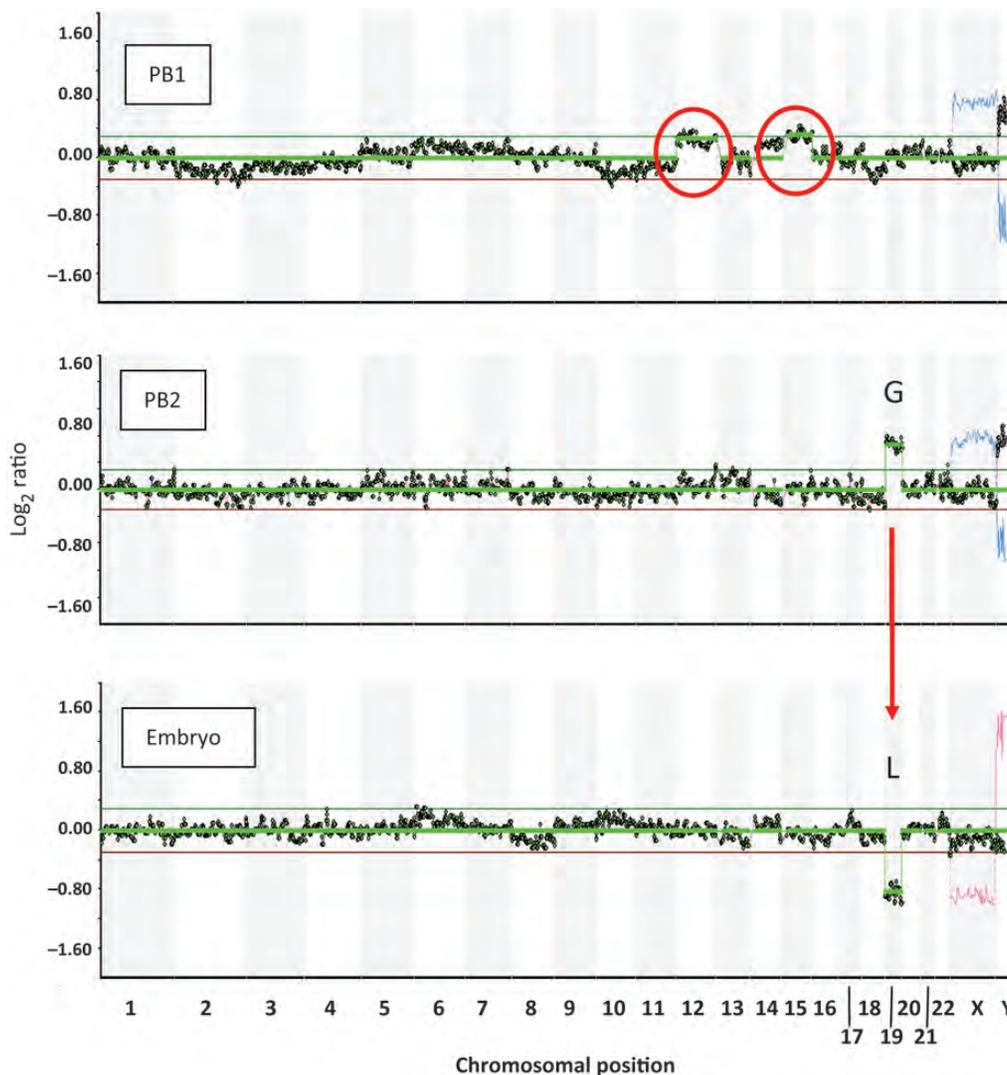


Figure 2 A second example of array CGH ratio plots for the first and second polar bodies (PB1 and PB2) and corresponding embryo (Embryo), also at the 8-cell embryo on Day 3 post-ICSI. There is only one error in MII, a chromatid gain (G) for chromosome 19 in PB2, resulting in loss (L) in the corresponding embryo. In addition, there are two possible gains for chromosomes 12 and 15 in PB1, which do not result in loss either in PB2 or the corresponding embryo (red circles). Note that although the software detects a significant shift for these chromosomes (bright green line) neither of these ratio changes approaches that of the internal X chromosome control as indicated by the blue (male) and pink (female) lines for sex mismatched DNA. Green and red horizontal lines represent the 95% confidence interval for normal copy number.

higher in PB1 when compared with PB2 (15 of 52, 29% versus 2 of 70, 3%, $P < 0.0005$). The reasons for this susceptibility to amplification bias are not clear. But, PB1 uniquely contains a haploid set of meiotic chromosomes in transition from metaphase to telophase/anaphase and the timing of biopsy may be critical. In the previous pilot study, both polar bodies were biopsied at the same time following ICSI (Handyside et al., 2012). Also, consistent with this, these small copy number changes were not evenly distributed between embryos with 2 embryos, for example, accounting for 7 of 17 (41%) (embryos 22 and 27; Table 1).

Of the four other embryos followed up at cleavage stages, three had only one or two copy number changes in the polar bodies (two each in PB1 and PB2) that had been recognized as small artefactual copy

number changes and predicted to have normal copy number in the corresponding embryos. However, other embryos with unambiguously normal copy number in the polar bodies were transferred and this resulted in two of the three pregnancies in this series of patients. These embryos were then followed up and were all confirmed to be euploid. This underlines the importance of assessing the size of the ratio shift before concluding that there is a copy number change, particularly if they occur in isolation, and where there is any ambiguity, it may be advisable to rebiopsy the embryo at cleavage or blastocyst stages to confirm the initial assessment. Finally, the fourth embryo which had a partial deletion of the short arm of chromosome 11 in PB2 was also rejected for transfer and was found to be euploid. As the cause of these *de novo* structural

abnormalities in meiosis is not yet understood, it is difficult to predict their inheritance in the embryo after completion of the two meiotic divisions. There were four other structural abnormalities in the polar bodies of the aneuploid embryos. Two of these also did not appear to affect the ploidy of the chromosome involved in the corresponding embryo. However, structural abnormalities in chromosome 2 and the X chromosome in PB2 of one embryo were associated with imbalance in the corresponding embryo and may represent an example of a *de novo* reciprocal translocation (data not shown).

Accurate false positive and negative rates allowing the calculation of sensitivity and specificity of aneuploidy detection will require follow-up of a larger series of both euploid and aneuploid embryos, ideally using molecular genetic markers to detect aneuploidy independently and to identify their origin. One approach would be to use karyotyping in which genome wide single nucleotide polymorphisms (SNPs) genotyping of the couple and whole genome amplification products of the polar bodies and embryos would enable the parental origin of each chromosome to be identified in polar bodies and embryo cells (Handyside *et al.*, 2010). Also, during the course of this study, a system in which samples and both sex-matched and sex-mismatched control DNAs are hybridized to separate arrays and compared *in silico* was introduced and has significantly improved the signal-to-noise ratio at the single-cell level. This should reduce the incidence of any ambiguous copy number changes. A large multicentre trial of polar body biopsy and array CGH in women of advanced maternal age, organized by the ESHRE, is currently underway (Geraedts, personal communication).

The pattern of segregation errors identified in the aneuploid embryos (Table 2) is closely similar to the previous pilot study (Handyside *et al.*, 2012). In the first meiotic division, MI, only one segregation error (3%) was caused by non-disjunction of a whole chromosome. All the other 37 errors were caused by pre-mature pre-division of chromatids and, as expected, 19 (51%) of these were balanced by random segregation of the single chromatids in the second meiotic division, MII. Also, because half of the MI errors were balanced, the proportion of maternal aneuploidies in the embryos originating in MI was much less than MII. In this small series of embryos, there were almost twice as many gains as losses in PB2 resulting in an excess of monosomies in the corresponding embryos (39 monosomies versus 24 trisomies). However, this difference was not significant and, in the larger pilot study, there was an excess of trisomies (Handyside *et al.*, 2012).

Recently, two studies have examined copy number in polar bodies by array CGH and followed this up with analysis of single cells biopsied at cleavage stages and by trophoctoderm biopsy at the blastocyst stage (Capalbo *et al.*, 2012; Kotze *et al.*, 2012). Kotze *et al.* (2012) demonstrated that following fertilization of donor oocytes, about half were aneuploid, but that the euploid/aneuploid status of the embryos did not correlate with embryo grading or their ability to develop to the blastocyst stage. Consistent with this, most of the embryos predicted to have aneuploidies of maternal meiotic origin in this study, developed normally to 6–10-cell stages on Day 3 post-ICSI and were confirmed to have between 1–6 aneuploidies.

Capalbo *et al.* (2012) compared array CGH results in polar bodies, single blastomeres and trophoctoderm cell biopsies in 21 embryos predicted to be aneuploid, which developed to the blastocyst stage in women over 40 years of age. In these embryos, there were 78 copy number changes in the polar bodies of which 73 (94%) accurately

predicted the copy number detected in the single blastomere, 4 (5%) were false positives and 1 (1%) false negative apparently balanced combination, which compares favourably with the results presented here. However, only 61 of 86 (71%) of the aneuploidies present in the single blastomeres were identified as of maternal meiotic origin when compared with 93% in our analysis. The most obvious explanation for this difference is that we analysed the whole cleavage stage embryo for accurate detection of aneuploidies present consistently in most, if not all, cells, whereas a single blastomere may be affected by chromosome mosaicism. Also, 20% of both maternal meiotic and paternal or post-meiotic aneuploidies, detected by polar body and blastomere analysis, respectively, were not present in the trophoctoderm samples [including 2 examples of trisomy rescue, one of which resulted in maternal uniparental disomy (UPD)], although there were 11 additional aneuploidies not detected at earlier stages. Clearly, these embryos were highly mosaic and combined molecular genetic analysis and array CGH analysis at all stages will be necessary to exclude technical errors and fully explain the evolution of these aneuploidies at these early stages.

Capalbo *et al.* (2012) conclude from their data that PGS for aneuploidy should be carried out as late as possible, i.e. by trophoctoderm biopsy at the blastocyst stage, if possible, using a technique that combines both copy number and molecular genetic analysis of the parental origin of the chromosomes to detect UPD. One possible approach would be to combine quantitative SNP genotype analysis with karyotyping (Handyside *et al.*, 2010). However, despite the technical challenges and the additional cost and effort, we believe that their data strongly makes the opposite case, particularly for routine application of array CGH. It is possible that their results reflect an exceptionally mosaic set of embryos, but even if confirmed in a larger series of embryos, it simply emphasizes the risks of transferring embryos with meiotic aneuploidies.

None of the possible outcomes for embryos inheriting meiotic aneuploidies, including uniform aneuploidy or aneuploid mosaicism with either biparental or UPD cells, is desirable. The large majority of aneuploidies are lethal and the risks of UPD include imprinting effects and reduction to homozygosity which could result in expression of recessive mutations. Thus, an approach targeted at the main cause of aneuploid pregnancy, i.e. errors in female meiosis, should, in general, be the most effective, except in the rare cases, where there is an elevated risk of paternal aneuploidies. Analysis of cells sampled from chromosomally mosaic embryos at later pre-implantation stages will always reveal a combination of meiotic aneuploidies and those arising in cleavage and the extent of any mosaicism in an individual embryo, and particularly the inner cell mass at the blastocyst stage from which the fetus is derived, can never be ascertained.

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Authors' roles

D.C., sample collection and preparation, acquisition, analysis and interpretation of data, manuscript preparation. E.T., sample collection

and preparation, acquisition, analysis and interpretation of data. K.E., polar body biopsy and cleavage stage embryo sampling and preparation. P.S., data analysis and interpretation. S.D., polar body biopsy and cleavage stage embryo sampling and preparation. M.M., clinical management, guidance and support. A.H.H., study design, data analysis and manuscript preparation.

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Conflict of interest

A.H.H. and P.S. are employed part time and full time, respectively, by BlueGnome Ltd, Fulbourn, Cambridge CB21 5XE, UK that manufactures microarrays and supplies software for pre-implantation genetics.

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