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Polar body array CGH for prediction of the status of the corresponding oocyte. Part I: clinical results

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BACKGROUND: Several randomized controlled trials have not shown a benefit from preimplantation genetic screening (PGS) biopsy of cleavage-stage embryos and assessment of up to 10 chromosomes for aneuploidy. Therefore, a proof-of-principle study was planned to determine the reliability of alternative form of PGS, i.e. PGS by polar body (PB) biopsy, with whole genome amplification and microarray-based comparative genomic hybridization (array CGH) analysis.

METHODS: In two centres, all mature metaphase II oocytes from patients who consented to the study were fertilized by ICSI. The first and second PBs (PB1and PB2) were biopsied and analysed separately for chromosome copy number by array CGH. If either or both of the PBs were found to be aneuploid, the corresponding zygote was then also processed by array CGH for concordance analysis.

RESULTS: Both PBs were biopsied from a total of 226 zygotes from 42 cycles (average 5.5 per cycle; range 1–15) in 41 couples with an average maternal age of 40.0 years. Of these, the ploidy status of the zygote could be predicted in 195 (86%): 55 were euploid (28%) and 140 were aneuploid (72%). With only one exception, there was at least one predicted aneuploid zygote in each cycle and in 19 out of 42 cycles (45%), all zygotes were predicted to be aneuploid. Fresh embryos were transferred in the remaining 23 cycles (55%), and one frozen transfer was done. Eight patients had a clinical pregnancy of which seven were evolutive (ongoing pregnancy rates: 17% per cycle and 30% per transfer). The ploidy status of 156 zygotes was successfully analysed by array CGH: 38 (24%) were euploid and 118 (76%) were aneuploid. In 138 cases complete information was available on both PBs and the corresponding zygotes. In 130 (94%), the ploidy status of the zygote was concordant with the ploidy status of the PBs and in 8 (6%), the results were discordant.

CONCLUSIONS: This proof-of-principle study indicates that the ploidy of the zygote can be predicted with acceptable accuracy by array CGH analysis of both PBs.

Key words: microarray CGH analysis / polar body / oocyte / zygote / PGS

Introduction

Until recently, fluorescent *in situ* hybridization (FISH) of blastomeres, biopsied at the cleavage stage, was thought to be the best method for screening human preimplantation embryos for numerical chromosome abnormalities, a major presumed factor causing low pregnancy rates in medically assisted reproduction. Initially, many low-level evidence studies suggested a favourable outcome of preimplantation genetic screening (PGS) of aneuploidy with FISH on implantation and pregnancy rates (Gianaroli *et al.*, 1999; Munne *et al.*, 2003, 2005,

2006). However, more recent evidence from randomized control trials (RCT) has not confirmed these initially promising findings. There are now at least 10 RCTs applied to both good-(Jansen et al., 2008; Mersereau et al., 2008; Staessen et al., 2008; Meyer et al., 2009) and poor-prognosis patients (Staessen et al., 2004; Stevens et al., 2004; Debrock et al., 2007; Mastenbroek et al., 2007; Hardarson et al., 2008; Schoolcraft et al., 2009). These studies have all shown that PGS using cleavage-stage biopsy and FISH does not improve the live birth rate compared with a control group, and some of these studies have shown harm or had to be terminated prematurely. The American Society of Reproductive Medicine, the British Fertility Society and the European Society of Human Reproduction and Embryology (ESHRE) have all concluded that PGS, as it is currently practiced, does not improve the live birth rates in patients with an advanced maternal age, recurrent implantation failure or recurrent pregnancy loss (Anderson, 2008; ASRM, 2008; Harper et al., 2011).

Many groups are investigating means to improve PGS, using advanced testing methods or testing at different stages of embryo development. The ESHRE PGS Task Force has chosen to evaluate array technology at the polar body (PB) stage for evaluation. In 2008, the ESHRE PGS Task Force sought proposals for a molecular test which had to be applicable on the first and second PB (PB1and PB2) (and zygotes for confirmation) and able to identify whole chromosome aneuploidy reliably within 12 h.

Only one prototype bacterial artificial chromosome (BAC) array technology was put forward. However, it had not been clinically validated. The ESHRE PGS Task Force therefore undertook a proof-of-principle study to investigate the feasibility and reliability of this methodology. The specific objectives of this study were to show that the analysis of both PBs could be completed within 12 h and to ensure the reliable identification of the chromosomal status of a zygote in at least 90% of PB biopsy attempts. The latter objective was evaluated by comparing the results obtained from the screened PBs with those from the zygote.

Materials and Methods

Selection of experimental method

All relevant vendors at the annual meetings of the ESHRE and the European Society of Human Genetics in 2008 were invited to offer a molecular test with the following requirements:

(1) It must be applicable at the single cell level for analysis of the PB1and PB2, and zygotes (for confirmation).
(2) It should reliably identify whole chromosome aneuploidy within 12 h after receiving the sample.
(3) It must deliver an accurate reportable result in at least 90% of biopsy attempts.

On 1 December 2008, a Task Force meeting was held in Geneva. At that time, BlueGnome was the only applicant with an offer meeting these specific requirements. It consisted of a BAC microarray-based 24 chromosome aneuploidy screening technology, originally developed for prenatal diagnosis. After its presentation, it was decided to apply BlueGnome's technology in this proof-of-principle study.

Selection of study centres

On the basis of their experience with PB biopsy, two study centres were selected: SISMER Bologna, Italy and the Department of Gynecological Endocrinology & Reproductive Medicine at the University of Bonn, Germany. Both participating centres had a documented record of success with the clinical application of PB biopsy (Magli et al., 2004, 2011; Landwehr et al., 2008; Montag et al., 2009). Only experienced and trained personnel were involved in performing the biopsies.

All data (patient and cycle data as well as array data) from both study sites (PBs and oocytes) were evaluated by an independent data analysis team at the University of Amsterdam.

Ethical approval

All patient materials were obtained and evaluated with informed patient consent and under approval from the Ethics Committees from both centres. The approval was given in Bologna by the Ethics Committee of the SISMER center and in Bonn by the Local Ethics Committee implemented for all medical research topics by the Medical Faculty at the University of Bonn. Patients had to sign an informed consent prior to entering the study

Patient inclusion

There were no restrictions with respect to the maternal age of the patients. Only ICSI cycles were included. Micro-epididymal sperm aspiration and testicular sperm extraction cycles were excluded, since they may be associated with a higher incidence of male gonosomal aberrations and thus could impact the concordance in zygote analysis.

Every treatment cycle included in the study was given a unique code. This code consisted of a centre code (BLQ for Bologna and BON for Bonn) together with a three digit number (001, 002, etc.). Patients were numbered according to their date of inclusion.

Biopsy

Biopsy of the PB1and PB2 was performed between 6 and 9 h after insemination by ICSI. Before the zygotes were biopsied, all adhering cumulus cells were removed. PB1and PB2 were simultaneously removed and separately processed for chromosomal analysis. In Bologna, mechanical opening of the zona pellucida was performed. In Bonn, PB biopsy was performed after laser-assisted opening of the zona pellucida. All manipulations of PBs and zygotes were performed in a safety cabinet. All amplifications were carried out in a class II Laminar flow cabinet.

Workflow

The study samples were processed according to the following steps:

(1) amplification;
(2) labelling;
(3) hybridization;
(4) washing and
(5) scanning and data analysis.

A clinical quality BAC microarray for the comparative hybridization of amplified sample DNA against an amplified commercial control was applied. Replication strategies and controls were used to deliver objective and unambiguous estimation of aneuploidy at the whole chromosome level.

For amplification, the SurePlex™ kit was used to generate microgram levels of DNA template from PBs and zygotes. Specific quality control (QC) criteria for sample quality (size) and quantity (nanodrop) were used to ensure that only specific amplifications were labelled, using a quality-controlled labelling system (random primer, Cy dye).

PB samples were hybridized against male control DNA and the resulting chromosome Y profiles were used as a QC check to determine the

specificity amplification/labelling by estimating how close profiles are to the expected chromosome Y nullisomy.

The microarrays were scanned by making use of an Innopsys 710A microarray scanner. This is an autofocus 3–40 μm resolution scanner, which can produce data of high quality (www.innopsys.com). The BlueFuse Multi-software 'one button-'automated image analysis was used to estimate copy number on the basis of multiple independent measurements on each chromosome. Visualization and reporting of aneuploidy was on a per chromosome basis. Only whole chromosome aneuploidies (gains and losses) were scored. The PBs with amplification but no diagnosis were determined inconclusive. Structural chromosome defects have been ignored for this analysis except for patients with a known maternal translocation ($n = 3$).

Before starting the study, the laboratory personnel at both biopsy centres were trained by BlueGnome to perform the microarray procedure and analysis. This training included all stages of the 24sure protocol, interpretation of QC data, software data analysis and reporting. Upon completion of training BlueGnome independently validated each laboratory. A series of 25 amplifications were completed in Cambridge, UK and distributed blindly to the study centres. Thereafter, a series of five amplifications followed by another set of amplifications and labellings were processed in the study centres and sent blinded to BlueGnome for processing and evaluation.

One technician was able to process the samples alone, but for the diagnosis the calling from two independent technicians was requested.

Chromosomal analysis of zygotes

To estimate the concordance of the data from the PBs and the corresponding zygotes, a blind analysis was carried out for those zygotes that were presumably aneuploid based on the result of the chromosomal analysis of their PBs.

Zygotes were also available for concordance testing under the following circumstances:

- If one of the two PBs failed to amplify and the zygotes were not transferred, we defined as abnormal a zygote with an aneuploid PB irrespective of not having a result from the other PB. Conversely, a zygote with a normal PB and no result in the other was defined as unknown;
- If an error occurred during meiosis I which was corrected in meiosis II (so-called balanced euploid or aneuploid oocytes) and if these zygotes were not transferred or frozen;
- If the results from the 24sure analysis were unclear and the zygotes were not transferred.

From all these zygotes, the genetic material was amplified and half of the amplified material (DNA) was sent to the other pilot study site for further processing (labelling and hybridization) and blind evaluation of its aneuploidy status under the code generated by the centre. The data obtained from such zygotes were sent to Amsterdam where the data of the zygote were matched with the data previously obtained from the corresponding PBs.

The concordance analysis

All data (patient and cycle data as well as array data) from both study sites (PBs and zygotes) were sent to Amsterdam for evaluation and data analysis. The concordance analysis was made on the basis of three ploidy categories euploid/aneuploid/unknown. In the three cycles with patients that had translocations, the concordance analysis was limited to the chromosomes not involved in the translocation. Discordant cases were reviewed to determine which were truly discordant and which were the result of technical artefacts.

Results

In the two study centres, a total of 41 patients undergoing ICSI treatment were included (Table I). They underwent 42 cycles. An average maternal age was 40.0 ± 2.9 years. The mean number of mature metaphase II oocytes obtained after ovarian hyperstimulation was 9.5. On average 5.5 of these reached the 2 pronuclei (2PN) stage (range 1–15). Biopsy of both PBs was successful in 225 out of 226 zygotes.

The workflow was finished in 12 h in 33/42 (79%) cycles, while for the remaining an extra hour was needed due to the higher number of cells to be analysed. Whole genome amplification (WGA) was achieved in 214/226 (95%) of PB1 and array comparative genomic hybridization (CGH) provided a result in 212: 90 (42%) were euploid and 122 (58%) were aneuploid (Table II). WGA was also achieved in 214/226 (95%) of PB2 and array CGH provided a result in 207 (92%): 65 (31%) were euploid and 142 (69%) were aneuploid. Some examples are given in Figs 1 and 2.

In 195 cases results from both PBs were obtained. These results revealed that the chromosomal status of the zygote (i.e. the chromosomal status of the oocyte) was euploid in 55 (28%) cases and aneuploid in 140 (72%) cases. In 41 out of 42 cycles, at least one zygote was predicted to be aneuploid, whereas in 19 out of 42 (45%) cycles all zygotes were predicted to be aneuploid. Thus, in 23 out of 42 cycles a fresh transfer was possible (total number of embryos transferred 37, average 1.6 per transfer). These transfers lead to seven clinical pregnancies of which six evolutive (three twin pregnancies and three singleton pregnancies). In addition, one frozen-thaw cycle was performed on two euploid oocytes, which were frozen at the 2PN stage. This cycle resulted in an ongoing singleton pregnancy. In total, this results in a clinical pregnancy rate of 19% (8/42) per cycle and 33% (8/24) per transfer and an ongoing pregnancy rate of 17% (7/42) per cycle and (7/24) 29% per transfer. The implantation rate was 26% per embryo transferred (10/39).

Table I Patient and treatment characteristics.

Patients					
Number of cycles (patients)	42				
Average age female	40.0				
Average age male	41.8				
Average number of oocytes	9.5				
Average number of MFII	7.5				
Average number of 2PN	5.5			Including cryocycles	
Total number of 2PN	233				
Average number transferred	0.9	Per cycle			
Cycles with transfer	23	55%	Per transfer	24	Per transfer
Pregnancy (+hCG)	7	17%	30%	8	33%
Clinical pregnancy	7	17%	30%	8	33%
Ongoing pregnancy	6	14%	26%	7	29%

Seven children have been born so far (one pair of twins and five singletons) and all are apparently healthy. Two of the five singleton births resulted from pregnancies in which two embryos implanted. In the first pregnancy one baby was born, while one fetus died at Week 26; the chromosomal analysis revealed a normal karyotype. In the other pregnancy one sac vanished.

Table II Ploidy status of PBs and oocytes.

		(%)	
PB1			
Total number biopsied	226		
Total number amplified	214	95	
Euploid	90	40	42% of 212 diagnosed
Aneuploid	122	54	58% of 212 diagnosed
Unknown	14	6	
PB2			
Total number biopsied	226		
Total number amplified	214	95	
Euploid	65	29	31% of 207 diagnosed
Aneuploid	142	63	69% of 207 diagnosed
Unknown	19	8	
All PBs			
Total number biopsied	452		
Total number amplified	428	95	
Euploid	155	34	37% of 419 diagnosed
Aneuploid	264	58	63% of 419 diagnosed
Unknown	33	8	
Predicted oocytes			
Total number biopsied	226		
Total number result	195	86	
Euploid	55	28	
Aneuploid	140	72	
Analyzsed oocytes			
Total number analyzsed	177		
Total number result	156	88	
Euploid	38	24	
Aneuploid	118	76	

A total of 177 untransferred zygotes were available for concordance analysis. A result was obtained in 156, of which 38 (24%) were euploid and 118 (76%) were aneuploid. Of these 156 zygotes, 18 could not be included in the concordance analysis because the diagnosis of the PB was unknown ($n = 5$), the ploidy of the oocyte could not be predicted because of compensated aneuploidy in PB1 and PB2 ($n = 7$), or the result of the zygote was obscured by cumulus cell contamination ($n = 6$). The presence of cumulus cells in the zona pellucida could affect the quality of results considerably. This was especially evident in the analysis of the latter six oocytes in which the low standard deviation of the clones along the mean line was indicative of possible external DNA contamination (Fig. 3). The presence of this contaminating normal DNA makes the specific gain and losses very subtle. Further evidence of possible maternal contamination is that the scan images of the six oocytes in which cumulus cell contamination was suspected were all apparently female. All of these oocytes had been prepared by Pronase treatment.

Of the remaining 138 oocyte/PB pairs, 130 (94%) were concordant and 8 were discordant. Table III shows the eight discordant cases; in seven cases where the PBs indicated aneuploidy and the zygote was euploid, and in one case where the PBs indicated euploidy and the zygote was aneuploid. In the latter case, the zygote was not transferred because it contained an unbalanced translocation. There was not a significant difference in the concordance rate between the two centres.

Discussion

This study was undertaken to determine whether biopsy of the PB1 and PB2 followed by a subsequent analysis of the complete chromosome complement of these PBs using array-based CGH enables a reliable, timely and accurate identification of the maternal contribution to the chromosomal status of the corresponding zygote.

Our first aim was to demonstrate that microarray analysis of both PBs could be completed within 12 h. We chose this time period as this would allow for fresh transfer in all countries around the globe,

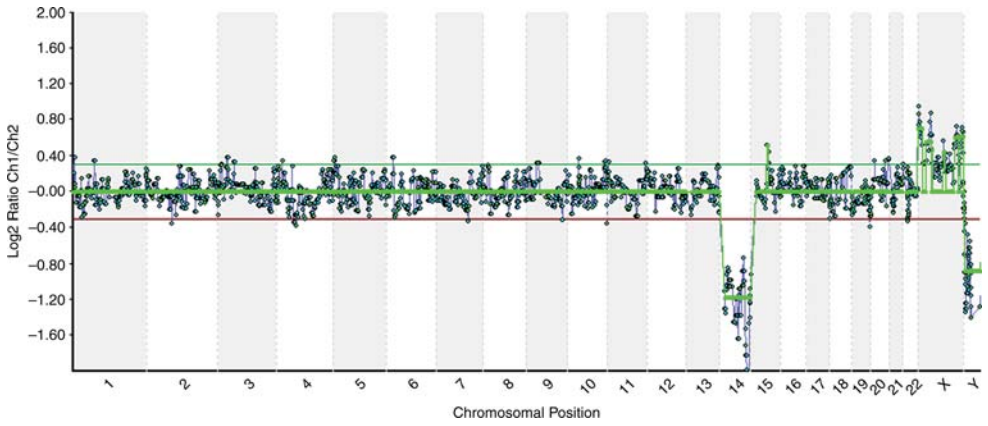

Figure 1 Results from a PB 1 carrying a loss of chromosome 14. All other non-sex chromosomes are within the bounds of normality.

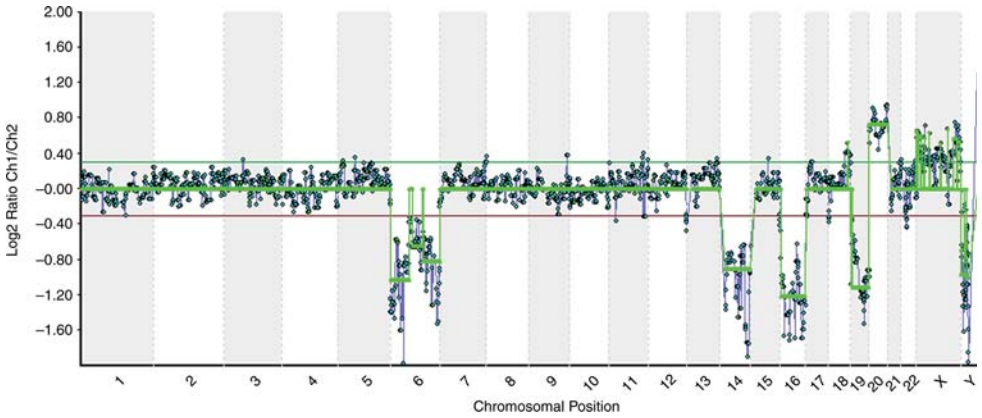

Figure 2 Polar body II with losses of chromosomes 6, 14, 16, 19 and gain of chromosome 20.

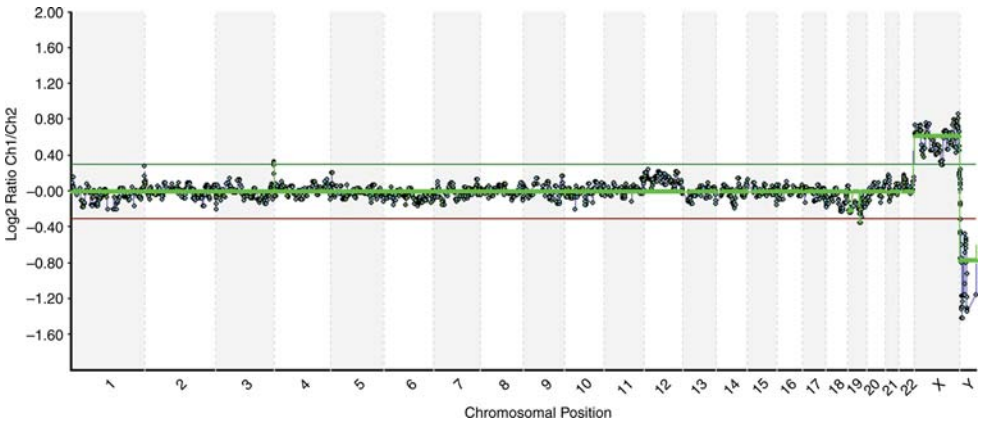

Figure 3 Possible contamination by cumulus cells in an oocyte that was predicted to be aneuploid, for chromosomes 12, 18 and 19. The contamination by foreign DNA is suspected due to the low, standard deviation of all clones and the faint shifts for the predicted aneuploid chromosomes 12 (gain), 18 (loss) and 19 (loss).

including those that allow only embryo selection at the zygote stage such as Germany and Switzerland.

The workflow took >12 h when there were more than six embryos. The speed of the workflow can be further increased by automation of labelling, hybridization and scanning, which will allow for the analysis of all embryos within a 12-h time frame. In countries where there are no restrictive laws in terms of timing of embryo selection, some steps in the workflow can be extended to an overnight procedure so that all of the steps of the workflow for which the presence of skilled personnel is necessary can be accommodated during normal working hours. Thus, the results for all embryos should be available on Day 2 of preimplantation development.

The second aim of our study was the reliable and accurate identification of the maternal contribution to the chromosomal status of the zygote in 90% or more of cases. Reasons for diagnostic problems were mainly failed amplification or hybridization, or inconclusive results because of high noise of the signals. Regarding the failed amplification, it was clear that special attention has to be paid to the transfer of the PB to the corresponding PCR tube, whereas for the following steps, a critical point is represented by the desiccation and resuspension of the pellets after labelling.

In this study, the rate of concordance between PBs and zygotes has been studied for all chromosomes. This was considered to be a key point for the evaluation of the procedure.

During the past 10–15 years, PGS has been extensively applied, mainly by FISH on blastomeres biopsied from cleavage-stage embryos. The reported data show that, depending on the method used, the error rate can vary between 2 and 50% (Munne *et al.*, 2002; Baart *et al.*, 2004; Li *et al.*, 2005; Colls *et al.*, 2007; Magli *et al.*, 2007; Gutierrez-Mateo *et al.*, 2011).

The concordance rate between the array CGH results for both PBs and that for the corresponding zygote was 94% (130/138). In seven of the eight discordant cases, one or both PBs were aneuploid and the zygote was euploid. The discordant cases might have originated from the compensation of aneuploidy by the sperm or from anaphase lagging, especially at meiosis II, leaving behind some chromosomes in the oocyte which would then be degraded to make the resulting zygote euploid. Spurious results might also be caused by fragmentation

Table III Analysis of eight discordant cases.

Case	Result from PB1 analysis	Result from PB2 analysis	Predicted oocyte ploidy	Evaluation of oocytes
1	Gain 17,20,21; Loss 13,22	Gain 13,22; Loss 17,20,21; partial 9[a]	Gain partial 9; Loss-	Euploid[d]
2	Gain 2,20; partial 15; Loss -	Gain -; Loss 15	Gain -; Loss 2,20; partial 15	Euploid[d]
3	Gain 21,X; Loss 15	Gain 15; Loss -	Gain -; Loss 21,X	Euploid[d]
4	Gain -; Loss-	Gain -; Loss 21,22	Gain 21,22; Loss -	Euploid
5	Gain -; Loss partial 1p, partial 9[b]	Gain -; Loss -	Gain partial 1p, partial 9; Loss -	Euploid[c]
6	Gain 11p; Loss 9,19p	Gain -; Loss 11p	Gain 9,19p; Loss -	Euploid for chr. 9[c]
7	Gain -; Loss 11q	Gain 11q; Loss 19q	Gain 19q; Loss -	Gain 19q; Loss 2,3,17,20,11p
8	Gain 13,14; Loss -	Gain -; Loss 14	Gain -; Loss 13	Euploid[d]

[a]The partial loss involved seven BAC clones and was therefore considered a relevant aneuploidy.
[b]The partial loss involved five BAC clones for chromosomes 1 and 7 for chromosome 9 and was therefore considered a relevant aneuploidy.
[c]Cases 5 and 6 were from a patient with a balanced translocation and the chromosomes involved in the translocation (46,XX,t(11;19);(11q.10;19p.10) were excluded from the concordance analysis for aneuploidy.
[d]In cases where for one or more chromosomes losses were observed in one PB and gains in another, the deviation of the losses/gains from the euploidy base line were evaluated. For the cases 1, 2, 3 and 8, the oocytes were predicted to be compensated euploid for the indicated chromosomes.

of PBs, although this was unlikely; with the time frame for biopsy, PB2 fragmentation was very infrequent. In most cases, it was actually possible to distinguish the PB2 from the PB1, due in part to the experience gained in biopsying oocytes and to the now-prevalent careful scoring of the PB1 morphology when doing ICSI. Therefore, relevant notes were taken in the working sheets and in cases of fragmented PB1, special care was taken to remove and transfer all fragments in the corresponding reaction tube. Comprehensive methodological aspects and detailed analysis of the results at the level of individual chromosome and chromatid abnormalities are published separately (Magli *et al.*, 2011).

The proportion of aneuploid zygotes in these patients was very high. The analysis of the data from the oocytes that were diagnosed as aneuploid by array CGH showed that 76.3% of the corresponding aneuploidies would have been detected using a conventional five-colour FISH probe set for chromosomes 13, 16, 18, 21 and 22. As a result 38 embryos classified as abnormal using array CGH would have been classified as normal using five-colour FISH as they involved aneuploidies not included in the usual FISH panel.

Furthermore, a very high incidence of multiple aneusomies was observed, although a relatively small number of aneusomies were potentially viable. The reasons for these might be truly biological [for example as a result of advanced maternal age or related to assisted reproduction technology (ART)] or technical (array-CGH-related). The analysis of PB2 adds significantly to achieve prediction of the oocyte ploidy. PB1 analysis alone only detects 70% of aneuploid oocytes and only half of the viable aneusomies. These results are in contrast to the data published by Sher *et al.* where the analysis of PB2 did not result in a gain in information following CGH (Sher *et al.*, 2007).

The BAC microarrays that have been used in this study are based on a form of CGH that was originally developed to analyse aneuploidy in cancer (Kallioniemi *et al.*, 1992). At that time both the test DNA and the reference DNA were labelled with two different fluorochromes and hybridized simultaneously onto normal male metaphase chromosomes. This method was first used on preimplantation embryos by Voullaire *et al.* (2000) and Wells and Delhanty (2000). In 2001, the first successful clinical application for aneuploidy detection after IVF was reported by Wilton *et al.* (2001). The application of this nowadays so-called 'classical' CGH is hampered by the amount of DNA and the time required for the analysis. The first clinical application of metaphase CGH on PBs was already reported in 2002 (Wells *et al.*, 2002). However, this method has never been used on a large-scale since then because it requires experience and is labour intensive. Therefore, the development of a simpler and more rapid CGH methodology was needed. The first clinical application of the combination of WGA of PB1 and BAC microarray analysis has recently been achieved by Fishel *et al.* who reported the birth of a normal healthy baby (Fishel *et al.*, 2010).

In conclusion, our study clearly demonstrates that chromosome aneuploidy of the oocyte can reliably and timely be predicted by array CGH analysis of both PBs. Aneuploidy appears the predominant cause of non-viability at least in this group of patients. Further progress, in the course of this project, will move the technique from the pilot phase to a clinical validation phase. The aim of this progress is not only to demonstrate that it is possible to have a reliable and accurate diagnosis on PBs but also that this approach is effective in improving ART results. The next question that needs to be answered is 'Does this method decrease the time to pregnancy by allowing the early transfer of euploid embryos and by avoiding the transfer of aneuploid embryos?' Given the large proportion of women having no euploid oocytes, a second question might be addressed: 'Can this method reliably predict which group of patients has a very low chance of pregnancy?' Only by testing these questions in a randomized clinical trial can the applicability and value of this approach be proved. The ESHRE PGS Task Force hopes to be able to address these important questions in a multicentre randomized trial to be started in 2011 based on the technology used in the pilot study.

Authors' roles

All authors designed the study. C.M. and M.M. performed the microarray experiments. S.R. performed the initial concordance analysis.

J.G. wrote the first draft of the manuscript. A.H.H., C.M., M.M. and S.R. completed the draft. All authors modified and improved it.

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Conflict of interest

A.H.H. after completion of this study, became a part-time employee of BlueGnome Ltd. who manufactured the arrays used in the study. All authors have completed the ICMJE conflict of interest disclosure form, and have no conflicts to declare.

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