| human reproduction | **ORIGINAL ARTICLE** *Reproductive genetics* |

Application of next-generation sequencing technology for comprehensive aneuploidy screening of blastocysts in clinical preimplantation genetic screening cycles

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STUDY QUESTION: Can next-generation sequencing (NGS) techniques be used reliably for comprehensive aneuploidy screening of human embryos from patients undergoing IVF treatments, with the purpose of identifying and selecting chromosomally normal embryos for transfer?

SUMMARY ANSWER: Extensive application of NGS in clinical preimplantation genetic screening (PGS) cycles demonstrates that this methodology is reliable, allowing identification and transfer of euploid embryos resulting in ongoing pregnancies.

WHAT IS KNOWN ALREADY: The effectiveness of PGS is dependent upon the biology of the early embryo and the limitations of the technology. Fluorescence *in situ* hybridization, used to test for a few chromosomes, has largely been superseded by microarray techniques that test all 24 chromosomes. Array comparative genomic hybridization (array-CGH) has been demonstrated to be an accurate PGS method and has become the *de facto* gold standard, but new techniques, such as NGS, continue to emerge.

STUDY DESIGN, SIZE, DURATION: The study consisted of a prospective trial involving a double blind parallel evaluation, with both NGS and array-CGH techniques, of 192 blastocysts obtained from 55 consecutive clinical PGS cycles undertaken during the period of September to October 2013. Consistency of NGS-based aneuploidy detection was assessed by matching the results obtained with array-CGH-based diagnoses. Primary outcome measure was accuracy of the chromosomal analysis; secondary outcome measures were clinical outcomes.

PARTICIPANTS/MATERIALS, SETTINGS, METHODS: Fifty-five patients (median age 39.3 years, range 32–46) undergoing PGS were enrolled in the study. All embryos were cultured to blastocyst stage; trophectoderm biopsy was performed on Day 5 of development or Day 6/7 for slower growing embryos. The method involved whole genome amplification followed by both NGS and array-CGH. The MiSeq® control software, real-time analysis and reporter performed on-board primary and secondary bioinformatics analysis. Copy number variation analysis was accomplished with BlueFuse Multi software.

MAIN RESULTS AND THE ROLE OF CHANCE: A total of 192 blastocysts were blindly evaluated with the NGS-based protocol. Paired comparison between NGS and array-CGH from individual embryos showed concordant results in 191/192 (99.5%) of the blastocysts tested. In total 4608 chromosomes were assessed, 211 (4.6%) of which carried a copy number imbalance. NGS specificity for aneuploidy calling (consistency of chromosome copy number assignment) was 99.98% (4333/4334; 95% confidence interval [95% CI]: 99.87–100) with a sensitivity of 100% (211/211, 95% CI: 99.25–100). Despite one discordant result, NGS specificity and sensitivity for aneuploid embryo calling (24-chromosome diagnosis consistency) were both 100% since the discordant sample presented several other aneuploidies. Clinical application of the NGS-based approach revealed 74/192 (38.5%) euploid blastocysts. Following transfer of 50 embryos in 47 women, 34 women had positive hCG levels: 30 pregnancies continued, confirmed by at least one fetal sac and heart beat (63.8% clinical pregnancy rate/embryo transfer), 3 were biochemical

and 1 miscarried. A total of 32 embryos implanted and led to the presence of a fetal sac (64.0% implantation rate). All pregnancies went to term resulting in the birth of 31 healthy babies.

LIMITATION, REASON FOR CAUTION: Although clinical results reported high pregnancy outcomes following transfer of screened embryos, further data and broad-based clinical application are required to better define the role of NGS in PGS. Before recommending widespread application, a randomized controlled trial confirming its clinical effectiveness is advisable.

WIDER IMPLICATION OF THE FINDING: This is the first study reporting extensive application of NGS-based comprehensive aneuploidy screening on embryos at blastocyst stage in a clinical setting versus array-CGH as test of reference. NGS has demonstrated a reliable methodology, with the potential to improve chromosomal diagnosis on embryos especially in terms of high-throughput, automation and ability to detect aneuploidy. NGS methodology may represent a valuable alternative to the other comprehensive aneuploidy screening techniques currently available.

STUDY FUNDING/COMPETING INTEREST(S): No external funding was sought for this study. Drs F.K. and C.-E.M. are full-time employees of Illumina, Inc., which provided NGS library and sequencing reagents for the study. All other authors have no conflicts to declare.

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Key words: comprehensive chromosome screening / preimplantation genetic screening / array-comparative genomic hybridization / next-generation sequencing / clinical outcomes

Introduction

Successful *in vitro* fertilization (IVF) is based in part on successful selection of viable embryos from a cohort following ovarian stimulation. For decades, selection of the most competent embryo(s) for transfer has been mainly based on morphological criteria, with the highest implantation rates observed with the use of optimal morphologic and developmental characteristics (Ebner *et al.*, 2003). However, it is well known that many women fail to achieve a pregnancy even after transfer of good quality embryos. One of the presumed causes is that such morphologically normal embryos are aneuploid.

A high rate of embryos produced *in vitro* present chromosomal aneuploidy, especially embryos derived from women of advanced reproductive age, and such embryos have reduced potential for achieving a viable pregnancy. Such abnormalities are recognized as the leading cause of implantation failure and spontaneous miscarriage (Macklon *et al.*, 2002; Lathi *et al.*, 2008), providing a likely explanation for the relatively low success rate observed during IVF treatments (Spandorfer *et al.*, 2004; Menasha *et al.*, 2005).

Several studies, assessing the correlation between blastocyst morphology and chromosomal status, demonstrated that normal preimplantation embryo development to the blastocyst stage does not correlate with euploidy (Fragouli *et al.*, 2008; Alfarawati *et al.*, 2011). In fact a significant proportion of aneuploid embryos were capable of achieving the highest morphologic scores, and some euploid embryos were of poor morphology. Hence, morphologic analysis of blastocysts cannot be relied on to ensure transfer of chromosomally normal embryos.

This poor correlation of morphology based embryo selection and chromosomal complement led to the introduction of preimplantation genetic screening (PGS), a technique enabling the assessment of the numerical chromosomal constitution of embryos before transfer. PGS has been proposed primarily as a method to improve embryo selection for patients with a poor prognosis for IVF success as a result of advanced maternal age, previous implantation failures or recurrent pregnancy loss (Wilton, 2002). Enhanced selection by PGS may provide a practical way to reduce substantially the risk of an adverse reproductive outcome related with the transfer of chromosomally abnormal embryos.

Initial studies on PGS, in the context of biopsy of single blastomeres from cleavage-stage embryos and the use of fluorescence *in situ* hybridization (FISH) technique, showed promising results and generated much hope. These findings encouraged the widespread use of PGS, providing an apparent opportunity to improve clinical outcome of IVF treatments by identifying and selecting chromosomally normal embryos for transfer. The persuasive rationale on PGS use was based on the assumption that excluding aneuploidy embryos from transfer should increase the implantation rate and decrease the risk of miscarriage (Lathi *et al.*, 2008).

However, while the premise behind PGS is widely accepted, its benefits with regard to live birth rate per started cycle have not yet been consistently demonstrated (Harper *et al.*, 2010; Mastenbroek *et al.*, 2011; Mastenbroek and Repping, 2014). In fact, a large number of prospective, randomized controlled trials (RCTs) have consistently failed to show any improvement in delivery rates using FISH-based PGS at cleavage stages (Mastenbroek *et al.*, 2011), although a recent RCT has reported a significant increase in live birth rates in patients with advanced maternal age (Rubio *et al.*, 2013). As a consequence of these studies, this methodology has become largely obsolete.

There are many possible reasons why the above clinical studies failed to deliver the expected improvements in IVF outcome. A possible explanation for this poor clinical performance has been attributed to the well-known limitations of the FISH technique, which screens for only a few chromosomes, most commonly observed in pregnancy loss and aneuploid deliveries, that are not necessarily the most relevant for early embryos (Harper and Harton, 2010; Harper *et al.*, 2010; Gutierrez-Mateo *et al.*, 2011). The first studies using comprehensive chromosome screening (CCS) technologies showed that aneuploidies may occur in any of the 24 chromosomes in preimplantation embryos. This indicates that aneuploidy screening of all chromosomes is necessary to determine whether an embryo is chromosomally normal (Wells *et al.*, 2008; Schoolcraft *et al.*, 2010; Treff *et al.*, 2010; Fiorentino *et al.*, 2011; Fiorentino, 2012; Gutierrez-Mateo *et al.*, 2011). Therefore, this may have led to reduced diagnostic accuracy and effectiveness of PGS with FISH technology, with elimination of any potential benefit of screening resulting from the transfer of reproductively incompetent embryos with aneuploidy for chromosomes which were not analysed and excluding incorrectly too

many chromosomally normal embryos (Harper et al., 2010; Scriven and Bossuyt, 2010).

Therefore, the focus in the PGS field has now shifted from Day 3 single blastomere biopsy to Day 5/6 trophectoderm sampling and the use of comprehensive chromosome screening technologies, in order to provide a more accurate assessment of the reproductive potential of embryos.

Among the different methodologies for comprehensive aneuploidy screening currently available for clinical use (Wells et al., 2008; Johnson et al., 2010; Treff et al., 2010, 2012; Fiorentino et al., 2011; Gutierrez-Mateo et al., 2011), array comparative genomic hybridization (array-CGH) was the first technology to be widely available (Wells et al., 2008). It has been extensively validated using cells of known genotype (Thornhill et al., 2014) and is now used extensively around the world.

The availability of robust and accurate methodologies allowing comprehensive aneuploidy screening has empowered a series of randomized controlled trials (Yang et al., 2012; Fiorentino et al., 2013; Scott et al., 2013). The results of these clinical studies provided evidence that aneuploidy screening of embryos can improve IVF clinical outcomes. As a consequence, it is expected that the clinical use of these technologies will increase steadily with the accumulating evidence of their clinical utility.

PGS for chromosome aneuploidy cannot create a healthy embryo or improve the health of an embryo. However, improved techniques for more accurate selection of embryos with the normal number of chromosomes for transfer has the potential to reduce the time in treatment to achieve a healthy live birth and reduce the risk of miscarriage or a profoundly disabled child due to an abnormal number of chromosomes.

Recent advances in next-generation sequencing (NGS) technologies have stimulated an increasing interest in its application in the field of reproductive medicine. In particular for PGS as an adjunct to IVF, because of the potential improvements that the technique may offer for detection of chromosomal aneuploidy in preimplantation embryos compared with current comprehensive aneuploidy screening methodologies (Handyside, 2013; Handyside and Wells, 2013; Martín et al., 2013; Rubio, 2014; Wells, 2014).

Chromosomal copy number assessment based on NGS may offer several advantages to array-CGH including: (i) reduced DNA sequencing cost made possible by high throughput sequencing technologies and the increasing number of samples that can be simultaneously sequenced during a single experiment; (ii) enhanced detection of partial or segmental aneuploidies as a result of the potential increase in chromosomal analysis resolution to a few megabases; (iii) increased dynamic range enabling enhanced detection of mosaicism in multicellular samples; (iv) the potential automation of the sequencing library preparation to minimize human errors, reduce hands-on time, and enable higher throughput and consistency (Handyside, 2013; Handyside and Wells, 2013; Treff et al., 2013; Yin et al., 2013; Fiorentino et al., 2014). We recently investigated whether NGS could be reliably applied for PGS (Fiorentino et al. 2014), by performing an extensive preclinical validation of a NGS-based 24-chromosome aneuploidy screening protocol. The study demonstrated that NGS is a robust methodology that may find a place in routine clinical application.

Although this approach offers exciting and potentially important advances towards improved PGS, its possible clinical effectiveness in PGS still remains unexplored.

Here, we present the findings of a prospective trial, performed on a cohort of 55 consecutive clinical PGS cycles, involving a parallel evaluation of embryos with both NGS and array-CGH techniques. The study aims to outline the potential for routine clinical use of the NGS methodology for comprehensive aneuploidy screening of preimplantation embryos at blastocyst stages of development.

Materials and Methods

Experimental design and clinical cases

This study represents the second phase of a strategy to validate the use of NGS for the clinical application of CCS of human embryos. The first phase involved a large preclinical validation study to determine the accuracy of the NGS-based 24-chromosome aneuploidy screening protocol (Fiorentino et al., 2014).

The study consisted of a prospective trial involving a double blinded parallel evaluation, with both NGS and array-CGH techniques, of embryos at blastocyst stage of development, obtained from clinical PGS cycles. Consistency of NGS-based aneuploidy detection was assessed matching the results obtained with array-CGH-based diagnoses, at the level of individual chromosome copy numbers for all 24 chromosomes of each sample tested and for the overall diagnosis of aneuploidy or euploidy. Discordant samples were subsequently re-evaluated as previously described (Fiorentino et al., 2010). Embryos were selected for transfer only if concordant results for both techniques were achieved.

Primary outcome measure was accuracy of the chromosomal analysis; secondary outcome measures included clinical outcomes.

The study population consisted of 55 consecutive patients planning to undergo PGS with trophectoderm (TE) biopsy (Fig. 1). All IVF cycles were performed at the European Hospital Reproductive Medicine Centre in the period between September and October 2013. Genetic testing was performed at Genoma PGD laboratory. During the study period CCS was offered to patients of advanced reproductive age, those with recurrent pregnancy loss or prior failed IVF cycles.

The study was approved by the Institutional Review Board of both European Hospital centre and GENOMA laboratory.

Case referrals and patient counselling

All the couples involved in the study were initially seen by a clinical geneticist. Genetic counselling consisted of reviewing the couple's clinical history, followed by an explanation of the PGS process, a discussion on the likely accuracy in terms of sensitivity, specificity, positive and negative predictive values of the procedure against the index result (array-CGH), potential benefits of testing and its limitations. A calculation of the possible genetic outcomes, the likely success rates, the possibility of having no embryos for transfer and the risk of misdiagnosis were also discussed. The patients were then referred to the collaborating IVF clinic to arrange the clinical aspects of the treatment.

Written informed consent was obtained from the each enrolled couple, as approved by the Institutional Review Board of both GENOMA and the collaborating IVF clinic, in which the possible risk of misdiagnosis was specified and confirmatory prenatal diagnosis for any ensuing pregnancy was recommended.

IVF and embryo biopsy procedure

Patients enrolled in this study were treated with a stimulation protocol and intracytoplasmic sperm injection (ICSI), as previously described (Greco et al., 2007, 2014).

On Day 3, a hole was made through the zona pellucida (ZP) of all cleaving embryos using a laser (Research Instruments, Cornwall TR11 4TA, UK) to facilitate blastocyst hatching. All embryos were cultured at 37°C, 6.0% CO_2, 5.0% O_2 and 89% N_2, in droplets of sequential culture media under oil and graded every day until blastocyst stage. On Day 5, all blastocysts
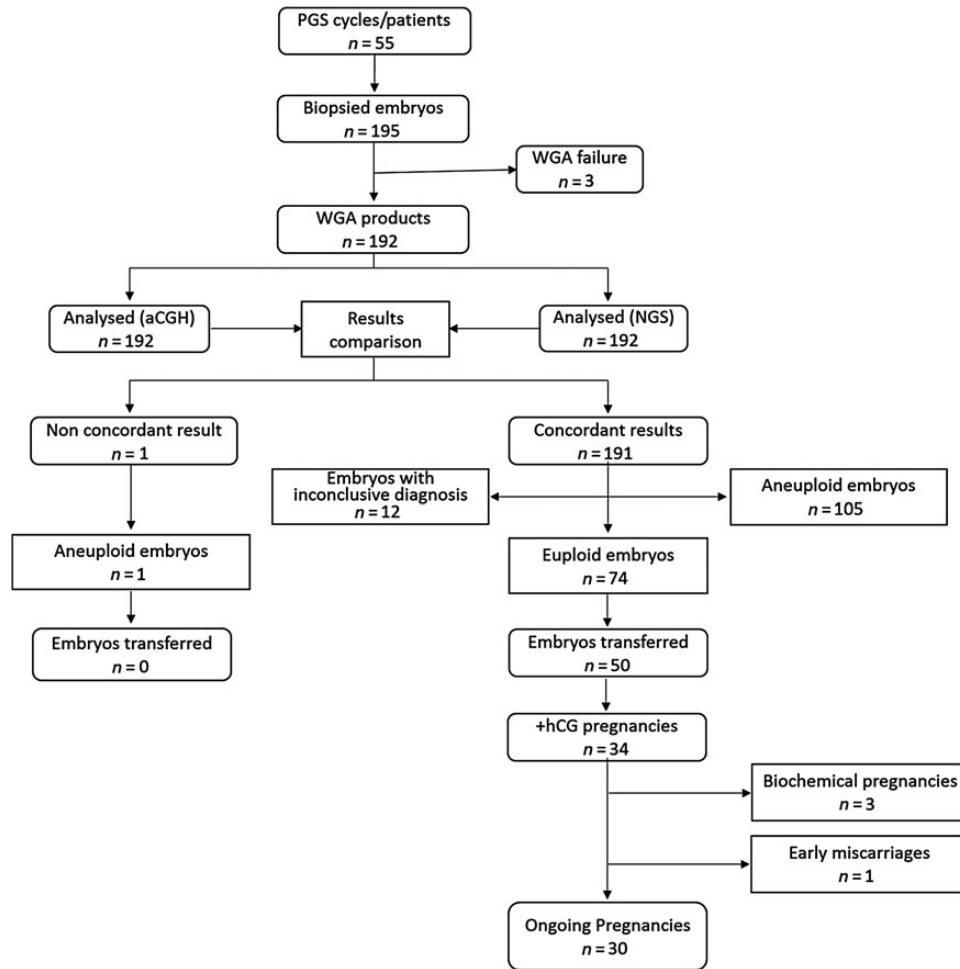
Figure 1 Recruitment and testing algorithms for participants. PGS, preimplantation genetic screening; WGA, whole genome amplification; aCGH, array comparative genomic hybridization; NGS, next-generation sequencing.

reaching at least an expansion of grade 3, with a distinct inner cell mass (ICM) and an adequate cellular trophectoderm, were biopsied (Gardner and Schoolcraft, 1999). The remaining slower growing embryos were reassessed on Day 6 and on Day 7 for possible TE biopsy with subsequent vitrification.

A sample of ∼6–10 TE cells was aspirated with a biopsy pipette (COOK, Ireland Ltd, Limerick, Ireland) and removed with the use of the laser. All biopsy procedures were performed in droplets of buffered medium (HEPES, Sage In-Vitro Fertilization, Inc., Trumbull, CT, USA) overlaid with mineral oil on the heated stage of a Nikon IX-70 microscope, equipped with micromanipulation tools. After biopsy, the TE cells were washed in sterile phosphate-buffered saline (PBS) solution (Cell Signalling Technologies, Beverly, MA, USA), placed in 0.2 ml PCR tubes containing 2 μl PBS and then transferred to GENOMA laboratory to be processed by array-CGH and NGS.

A maximum of two fresh euploid blastocysts were selected for transfer on the morning of Day 6. Euploid embryos biopsied in the late of Day 5 or on Day 6 or 7 were transferred in a subsequent natural frozen embryo transfer (FET) cycle. Euploid blastocysts were selected for transfer based on morphological score.

Cell lysis and whole genome amplification

For whole genome amplification (WGA), TE cell samples and negative controls were first lysed and genomic DNA was randomly fragmented and amplified using the SurePlex DNA Amplification System (Illumina, Inc., San Diego, CA, USA), according to the manufacturer's protocol. This proprietary single tube technology is based on random fragmentation of genomic DNA and subsequent amplification by PCR utilizing flanking universal priming sites as previously described (Alfarawati et al., 2011; Fiorentino et al., 2011; Yang et al., 2011).

Briefly, biopsies collected in 2.5 μl of 1 × PBS were lysed using 2.5 μl of SurePlex cell extraction buffer and 5 μl of the SurePlex Extraction cocktail master mix and incubation at 75°C for 10 min followed by incubation at 95°C for 4 min. The random fragmentation of genomic DNA was done by adding 5 μl of SurePlex Pre-amplification cocktail to the lysed biopsy samples or to genomic DNA controls and incubating the mixture according to the following protocol: one cycle of 95°C for 2 min, followed by 12 cycles of 95°C for 15 s, 15°C for 50 s, 25°C for 40 s, 35°C for 30 s, 65°C for 40 s and 75°C for 40 s, followed by a hold at 4°C. Thereafter, 60 μl of freshly pre-pared Sureplex Amplification cocktail was added to the 15 μl of synthesis product in each reaction tube. Resulting mixtures were amplified according to the following thermal cycler programme: one cycle of 95°C for 2 min, followed by 14 cycles of 95°C for 15 s, 65°C for 1 min and 75°C for 1 min, followed by a hold at 4°C. To determine the success of the amplification, 5 μl of each amplified sample plus 5 μl gel loading buffer were examined by electrophoresis on a 1.5% agarose 1 × TBE gel.

Array-CGH analysis

WGA products were processed with 24sure V3 microarrays (Illumina, Inc.), according to the manufacturer's protocol. Briefly, amplified samples, controls and some reference DNAs (415205-PK, Illumina, Inc.) were labelled with Cy3 and Cy5 fluorophores using random primers of the 24sure V3 Pack (408702-PK, Illumina, Inc.) which contains the reagents needed to perform an assay, including: 24sure V3 arrays, Fluorescent Labelling System [dCTP] and COT Human DNA. Every batch of biopsied samples requires hybridization of four labelled reference DNA samples; two male and two female. These were compared *in silico* with the intensities from biopsied sample hybridizations run at the same time in the same batch. The resulting labelling mixes were combined and co-precipitated with COT Human DNA in preparation for hybridization. Labelled DNA was resuspended in dextran sulphate hybridization buffer and hybridized under cover slips to 24sure V3 slides (Fiorentino *et al.*, 2014). Thereafter, the labelled products were hybridized to 24sure V3 slides and washed to remove unbound labelled DNA. A laser scanner was used to excite the hybridized fluorophores read and store the resulting images of the hybridization, as described elsewhere (Fiorentino *et al.*, 2011).

BlueFuse Multi software was developed to enable the analysis of the 24sure V3 experiments, including the automated creation of a reference database, using a single batch import file. The analysis of 24sure single channel experiments was fully automated and proceeded in a similar way to all BlueGnome microarrays. The software automatically combines the data from the single channel sample experiments with both male and female references from the hybridized reference subarrays, to produce a single fused result compared with a sex matched and a mismatched reference. Once a specific amplification was observed (i.e. low autosomal noise), autosomal profiles were assessed for gain or loss whole chromosomal ratios using a $3 \times SD$ assessment, greater than ± 0.3 log2 ratio call, or both. To pass hybridization quality control, female samples hybridized with a male reference DNA (sex mismatch) had to show a consistent gain on chromosome X and a consistent loss of chromosome Y (Gutierrez-Mateo *et al.*, 2011; Fiorentino *et al.*, 2011).

NGS analysis

Libraries were prepared at GENOMA Laboratory using the VeriSeq PGS workflow (Illumina, Inc.). DNA 'indexing' (Knapp *et al.*, 2012) was performed in order to simultaneously analyse embryos from different patients, using the Nextera XT 96 − Index Kit (Illumina, Inc.).

During the library preparation step, the input DNA is tagmented (tagged and fragmented) by the Nextera™ XT transposome. The Nextera transposome simultaneously fragments the input dsDNA and adds adapter sequences to the ends, allowing amplification by PCR in subsequent steps. The Nextera DNA Library Preparation Kit-PGS uses an engineered transposome to simultaneously fragment and tag ('tagment') input double-stranded DNA, thereby creating a population of fragmented nucleic acid molecules which comprise unique adapter sequences at the ends of the fragments. A limited-cycle PCR reaction uses these adapter sequences to amplify the insert DNA. The PCR reaction also adds index sequences on both ends of the DNA, thus enabling dual-indexed sequencing.

Briefly, WGA SurePlex template products were purified using the Zymo DNA Clean & Concentrator (Zymo Research Corporation, Irvine, CA, USA) and quantified using the Qubit® dsDNA HS Assay Kit (Life Technologies Corporation, Grand Island, NY, USA) because common contaminants such as ssDNA, RNA and oligonucleotides are not substrates for the VeriSeq PGS workflow. One nanogram of quantified dsDNA template at 0.2 ng/μl was added to 5 μl of Amplicon Tagmentation Mixture (ATM) and 10 μl of Tagmentation DNA Buffer (TD). The tagmentation step was carried out at 55°C for 5 min and hold at 10°C. The resulting tagmented mixture was neutralized by adding 5 μl of proprietary neutralization buffer (NT). Post-homogenization, the Tagmentation plate was held at room temperature for 5 min.

The tagmented DNA was amplified via a limited-cycle PCR programme (one cycle of 72°C for 3 min, followed by 12 cycles of 95°C for 10 s, 55°C for 30 s and 72°C for 30 s, one cycle at 72°C for 30 s, followed by a hold at 4°C after the adding of 5 μl of index 1 (i7), 5 μl of index 2 (i5) and 15 μl of Nextera PCR Master Mix (NPM) to each well.

PCR product clean-up used AMPure XP beads (A63881, Beckam Coulter, Brea, CA, USA) to purify the library DNA with no salt carryover, providing a size selection step that removes short library fragments including index 1 (i7) and index 2 (i5) from the population. Using a multichannel pipette, 45 μl of the PCR product was transferred to 96-well storage plates (AB0859, Fisher Scientific) containing 45 μl of AMPure XP beads. Sealed plates were mixed using a microplate shaker (444−7016, VWR) at 1800 rpm for 2 min, then incubated at room temperature without shaking for 5 min. Thereafter, the plate was placed on a magnetic stand (AM10027, Life Technology) for 2 min or until the supernatant cleared. While the plates were kept on the magnetic stand, the magnetic beads were washed twice with 200 μl of freshly prepared 80% ethanol (E7023, Sigma). Purified libraries were eluted with 50 μl of the Nextera XT Resuspension Buffer.

Single-end, dual index 36 base pair reads (1 × 36 donor insemination) sequencing was performed at GENOMA Laboratory following the Illumina v2 chemistry workflow on a MiSeq® (Part# SY-410-1003, Illumina, Inc.), using the MiSeq Reagent Kit v2 kit (Illumina, Inc.) which contains the ready to load on-board clustering and SBS chemistry reagents. The Nextera XT bead normalized indexed samples were multiplexed in 16 multiplexed library pools.

The sequenced samples had an average of 1 088 466 valid reads ('passing filter', SD = 539 614). Reads were aligned to the human genome hg19 using *bwa* (Li and Durbin, 2009) within the MiSeq Reporter Software. Bash scripting, BEDtools (Quinlan and Hall, 2010) and SAMtools (Li *et al.*, 2009) were used to remove unmapped reads, duplicate reads, non-unique reads, reads with low mapping scores and reads with an edit distance greater than one. The following bioinformatics analysis was accomplished with a pre-release version of BlueFuse Multi for NGS (Illumina, Inc.). Each chromosome was divided into intervals each approximately covering 1 Mb of sequence. Filtered reads from each sample were then mapped into the corresponding chromosome interval or bin. As previously described (Fiorentino *et al.*, 2014) the count data in each bin was normalized using GC content, and *in silico* reference data in order to remove bias, and copy numbers were determined using of a combination of a Gaussian probability function (PDF; with copy number states 0−4 and a standard deviation of 0.33) and thresholding. The copy number state with the highest probability for a chromosome was used unless the distance to the next most probable copy number was >0.011. In that case, the median value of the most likely copy number states of all bins of a chromosome was used, set to a gain when >2.5 and to a loss when <1.5.

Classification of results, concordance analysis, sensitivity and specificity assessment

NGS and array-CGH results were defined as previously described (Fiorentino *et al.*, 2011, 2014). Briefly, for array-CGH, trisomy (partial or full) was defined as a shift of the clones for the specific chromosome towards the green line (gain) on the whole chromosome BlueFuse Multi (BFM) plots. On the contrary, a monosomy (partial or full) was defined as a shift towards the red line (loss) of the BFM plots (Fig. 2, upper panels). For array-CGH, an 'inconclusive' result was assigned for a given chromosome when the ratio was below $3 \times SD$ or/and ± 0.3 log2 ratio call.

For NGS results, gains (partial or full) and losses (partial or full) were defined as a shift of the dots above and below the copy number state of 2.5 or 1.5, respectively, and detected as horizontal green bars (Fig. 2, lower panels). Inconclusive results were assigned to each chromosome when the reported copy number was between 2 and 2.5 or 2 and 1.5.
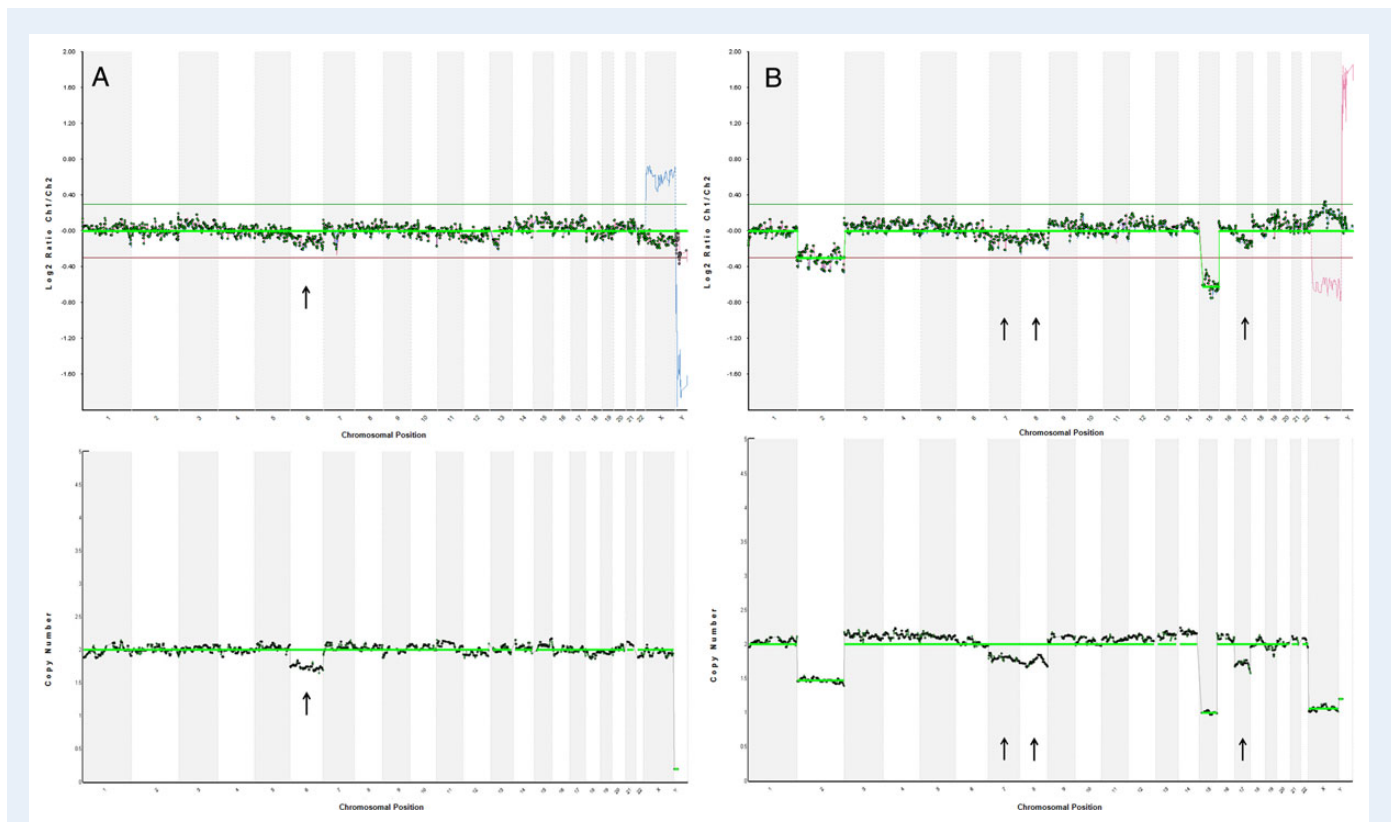
Figure 2 Examples of array comparative genomic hybridization (array-CGH) and next-generation sequencing (NGS) results. Top: results from array-CGH analysis. Bottom: results from NGS-based 24-chromosome aneuploidy screening analysis obtained from the same WGA product as shown in the upper panel. Each NGS graph in the bottom panel indicates the copy number assignments (0, 1, 2, 3, or 4) on the *y*-axis and the chromosome number on the *x*-axis. Gains and losses are seen as a shift of the dots above and below the copy number state of 2.5 or 1.5, respectively, and detected as horizontal green bars. Inconclusive results are assigned to each chromosome when the reported copy number is between 2 and 2.5 or 2 and 1.5. (**A**) Embryo showing an atypical loss for monosomy 6 that was below the automatic calling signal of the BlueFuse Multi Software (sample no. 59, Supplementary Table SI). The result was classified as 'inconclusive'. (**B**) Embryo showing aneuploidy for chromosomes 2 (monosomy 2) and 15 (monosomy 15), and atypical losses on chromosomes 7, 8 and 17 (sample no. 153, Supplementary Table SI). Black arrows indicate chromosomes with atypical losses.

Copy number calls automatically generated by the NGS pipeline and Blue-Fuse Multi were assessed manually and compared for sample ploidy status and chromosome ploidy status obtained with array-CGH.

Concordance of the NGS results (index) in respect to the array results (reference) was calculated using classifications as true positive (TP, gain or loss detected), true negative (TN, euploidy status confirmed), false negative (FN, gain or loss missed), or false positive (FP, incorrect gain or loss called).

To assess the reliability of NGS for aneuploidy detection, the sensitivity, specificity, positive and negative predictive values of the test were calculated as follow (Bossuyt, 2008):

Specificity: No. of True Negatives/(No. of True Negatives + No. of False Positives)

Sensitivity: No. of True Positives/(No. of True Positives + No. of False Negatives)

Positive predictive value: No. of True Positives/(No. of True Positives + No. of False Positives)

Negative predictive value: No. of True Negatives/(No. of False Negatives + No. of True Negatives)

The sensitivity is the proportion of embryos with an aneuploid (abnormal) array-CGH result that have an aneuploid NGS result. The specificity is the proportion of embryos with a euploid array-CGH result that have a euploid NGS result. The positive predictive value (PPV) is the proportion of aneuploid NGS results which are correct, and the negative predictive value (NPV) is the proportion of euploid (normal) NGS results which are correct.

Embryos were diagnosed as 'aneuploid' if the chromosomal copy number measures deviated from the default copy number. Euploidy was defined to be any multiple of the haploid chromosome number ($n = 23$) and therefore is not always normal (diploid). It is worth noting that some abnormal euploid embryos (e.g. $3n = 69$, $4n = 92$) may not be differentiated from normal diploid embryos ($2n = 46$). An 'inconclusive' diagnosis was assigned for those embryos with a pattern differing from embryos defined as normal (diploid) or abnormal (aneuploid).

Clinical data and definitions

The number of fertilized (two pronuclei) oocytes and the number of biopsied embryos were calculated on the basis of the total number of mature injected oocytes. The absence of an identifiable pregnancy on ultrasound examination following a positive pregnancy test was termed 'biochemical pregnancy loss' (Farquharson et al., 2005). Clinical pregnancy was defined as ultrasound demonstration of a gestational sac at 7 weeks after embryo transfer. Miscarriage was classified as 'early' (<12 weeks post embryo transfer) or 'late' (>12 weeks post embryo transfer). Implantation rate and ongoing implantation rate were defined as the number of gestational sacs per transferred embryo (expressed as a percentage), and number of fetuses with fetal

cardiac activity beyond 20 weeks of gestation per transferred embryo (expressed as a percentage), respectively.

Ethical approval

All the centres participating in the study have obtained ethical approval.

Results

Fifty-five patients (median age 39.3 years, range 32–46, Supplementary Fig. S1) undergoing PGS were enrolled in the study (Table I); 45 (median age 39.5 years, range 38–46) were with indication of advanced maternal age and 10 (median age 35.8 years, range 32–37) were patients with repeated implantation failure.

A total of 629 oocytes were collected (range 3–22 per oocyte retrieval), 512 (81.4%) of them were mature metaphase II stage, 410 (80.1%) fertilized normally (range 2–18 bipronucleate embryos per cycle), resulting in 195 embryos (median number per cycle 4; range

Table I Characteristic of patients involved in the study and clinical outcomes.

No. of couples treated	55
Mean female age in years (SD)[1]	39.9 ($\pm$ 2.4)
No. of PGS cycles performed	55
Indication	
Advanced maternal age ($\geq$ 38 years)	45 (81.8%)
Repeated implantation failures	10 (18.2%)
No. of oocytes retrieved	629
No. of mature oocytes injected[2]	512 (81.4%)
No. of oocytes fertilized[3]	410 (80.1%)
No. of embryos biopsied	195
Mean per cycle (SD)[4]	3.5 ($\pm$ 2.0)
No. of embryos analysed	195
No. of embryos with a WGA failure	3 (1.5%)
No. of embryos diagnosed	192 (98.5%)
No. of euploid blastocyst transferred	50
Mean (SD)	1.1 ($\pm$ 0.2)
No. of embryo transfers	47 (85.5%)
No. of +hCG pregnancies	34 (72.3%)
No. of biochemical pregnancies	3
No. of early miscarriages	1
Clinical pregnancy rate per ET (*N*)	63.8% (30)
No. of fetal sacs	32
No. of fetal sacs with heart beats	31
Implantation rate	64.0%
Ongoing implantation rate	62.0%
No. of pregnancies went to term	30
No. of babies born	31

ET, embryo transfer; PGS, preimplantation genetic screening; WGA, whole genome amplification; *N*, Number of clinical pregnancies.
[1]Median age (years): 39.3 (range 32.5–46.1).
[2]Calculated on no. of oocytes retrieved.
[3]Calculated on no. of mature oocytes injected.
[4]Median number of embryo biopsied per cycle: 4 (range: 1–10).

1–10) that reached blastocyst stage and were biopsied (Table I). WGA was successful in 192 of 195 (98.5%) TE biopsies. The resulting amplification failure rate was 1.5% (3/195) of the cells (Fig. 1).

A total of 192 embryos were blindly assessed with both array-CGH and the NGS-based 24-chromosome aneuploidy screening protocol. A normal (diploid embryo) BFM profile was observed in 74/192 (38.5%) of the embryonic cells (TE samples) with positive WGA. In 106 (55.2%) samples, one or more aneuploidies were detected, accounting for a total of 211 different aneuploid chromosomes, including 80 (37.9%) trisomies, 90 (42.7%) monosomies and 41 (19.4%) segmental imbalances. Twenty out of 106 aneuploid embryos presented atypical gains and/or losses for one or more chromosomes. These gains and/or losses, accounting for 38 chromosomes, were below the automatic calling signal of the BlueFuse Multi Software; the results were thus classified as 'inconclusive'. After manual assessment, these embryos were diagnosed as aneuploid (Table II).

Twelve out of 192 (6.3%) embryos presented atypical gains and/or losses on one or more chromosome, but had no aneuploidy on other chromosomes. After manual assessment, the results of these samples were classified as 'inconclusive' (Table II). The NGS and array-CGH BFM plots for these embryos were concordant (12/12, 100%). Examples of such NGS results are shown in Fig. 2. The details of karyotype predictions are included in Supplementary Table SI.

The NGS results were then compared for consistency with those obtained by previously established array-CGH methodology. Paired comparison between the two techniques from individual embryos showed concordant results for 191/192 (99.5%, 95% CI 96.8–99.9) blastocysts. A single embryo produced discordant results, consisting in an apparent false positive call by NGS for monosomy 22, which was later confirmed at GENOMA Laboratory using QF-PCR. However, this single discordant sample presented concordant NGS and array-CGH on two segmental imbalances, two chromosomal aneuploidies and inconclusive results on four chromosomes (Fig. 3), making this sample non-transferable by both methodologies.

Results from all of the remaining chromosomes for all of the remaining samples were consistent, including regions of segmental imbalances,

Table II Comprehensive aneuploidy screening results from the embryos investigated.

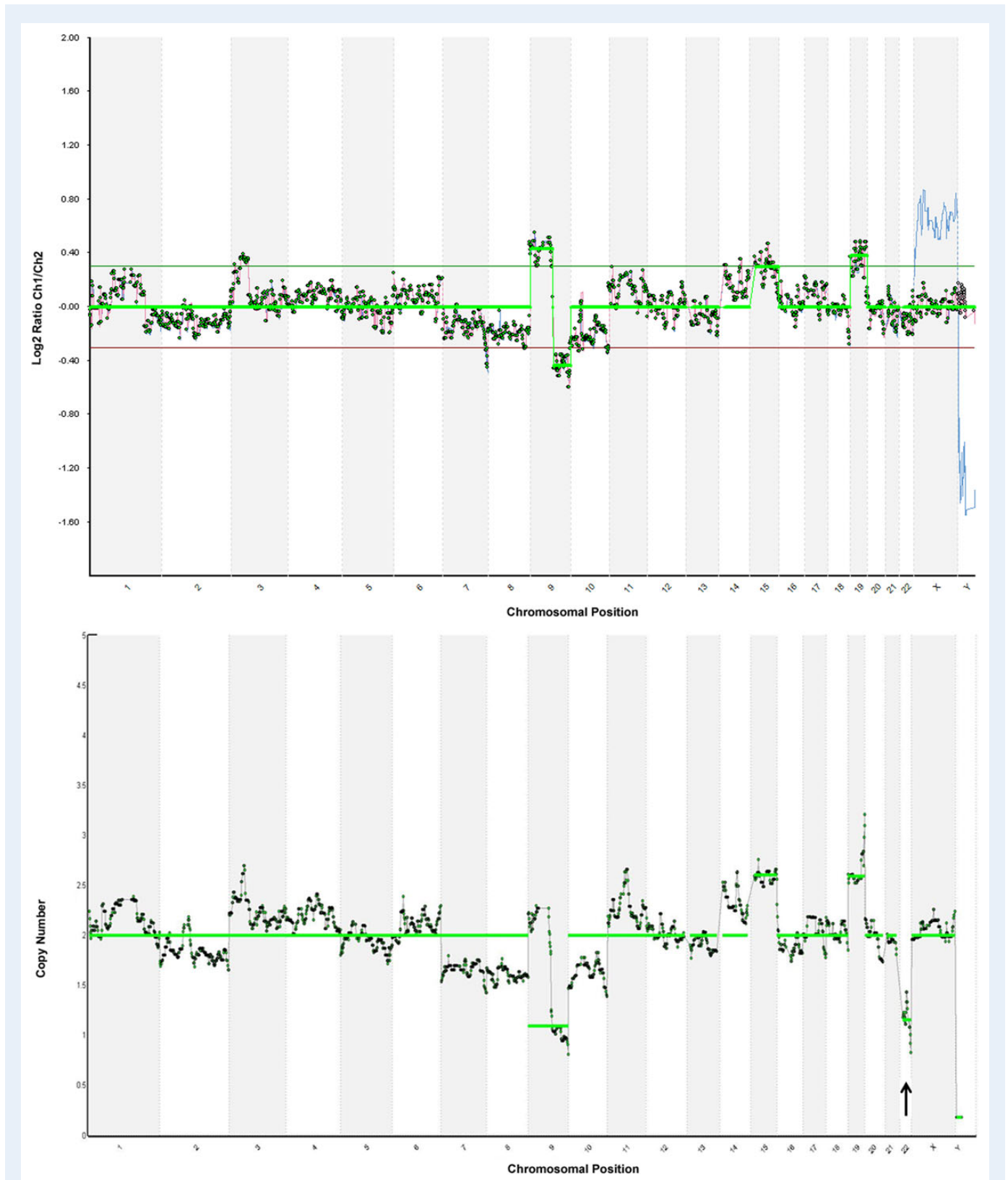| Samples	No. (%)
No. of embryos diagnosed	192
Euploid	74 (38.5)
Aneuploidy	106 (55.2)
Inconclusive	12 (6.3)
No. of chromosomes assessed	4608
No. of chromosomes with a conclusive diagnosis	4545
No. of chromosomes with an inconclusive diagnosis	63
Euploid	4334 (95.4)
Aneuploidy	211 (4.6)
Trisomies	80 (37.9)
Monosomies	90 (42.7)
Segmental imbalances	41 (19.4)

Figure 3 Graphic representation of copy number changes observed in the embryo that produced discordant results, consisting in a false positive call by next-generation sequencing (NGS) for chromosome 22 (monosomy 22). Top: array comparative genomic hybridization (array-CGH) graphic representation of copy number changes, showing aneuploidy for chromosome 15 and 19 (trisomy 15 and 19), inconclusive results (*) for chromosomes 7, 8, 10, and 14, segmental gains on chromosome 3 and 9, and a segmental deletion on chromosome 9 − (+3p, 7*, 8*, +9p, −9q, 10*, 14*, +15, and +19). Bottom: NGS graphic representation of copy number changes, showing a discordant aneuploidy for chromosome 22 (monosomy 22) (black arrow) − (+3p, 7*, 8*, +9p, −9q, 10*, 14*, +15, +19 and −22).

which were reliably identified with a segmental imbalance as small as 14.7 Mb in size. We only reported segmental imbalances over 5 Mb in size.

There were no false negative diagnoses for aneuploid chromosomes or embryos, or inaccurate predictions of gender.

In total 4608 chromosomes were assessed, 63 of which presenting atypical gains or/and losses were classified as inconclusive results and were not included in the concordance analysis. Of the 4545 chromosomes with a conclusive diagnosis, 211 resulted with a copy number imbalance (Table III). NGS specificity for aneuploidy call (consistency of chromosome copy number assignment) was 99.98% (4333/4334; 95% CI 99.87–100) with a sensitivity of 100% (211/211, 95% CI: 98.25–100). Despite one discordant result, NGS specificity and sensitivity for aneuploid embryo calling (24-chromosome diagnosis consistency) were both 100% since the discordant sample presented several segmental and chromosomal aneuploidies (Supplementary Table SI; Fig. 3). With a prevalence of 58.9% (106/180), the predictive value of the NGS-based 24-chromosome aneuploidy screening protocol was 100% (95% CI 95.09–100%) for a normal (74/74) and 100% (96.55–100%) for abnormal (106/106) index results (Table III).

Euploid embryos suitable for transfer were identified in 47 of the 55 cycles (85.5%—Table I). In 8 PGS cycles, embryo transfer was cancelled because only aneuploid embryos were identified. Following transfer of 50 embryos in 47 transfer cycles (mean ± SD embryo transfer number 1.1 ± 0.2, range 1–2), 34 women (mean age 38.5 ± 2.1 years, range 33–42) had positive hCG levels (72.3% positive pregnancy rate per embryo transfer); 30 pregnancies continued, confirmed by at least one fetal sac and heart beat (63.8% clinical pregnancy rate per embryo transfer), three were biochemical pregnancies only and one miscarried at the 9th week of pregnancy. Follow-up of the result with karyotyping of the product of conception was not possible. A total of 32 embryos implanted and led to the presence of a gestational sac (64.0% implantation rate), resulting in 31 fetuses with cardiac activity (62.0% ongoing implantation rate). Thirty pregnancies went to term resulting in the birth of 31 healthy babies.

Discussion

This is the first study reporting extensive application of NGS-based comprehensive aneuploidy screening on embryos at blastocyst stage derived from PGS cycles, demonstrating that NGS is a reliable methodology allowing identification and transfer of euploid embryos resulting in ongoing pregnancies.

This study represents the second of a three-phase strategy to validate the use of NGS for comprehensive aneuploidy screening as a preclinical step towards its routine use in the diagnosis of chromosomal aneuploidy on embryos.

The first phase, involving a large preclinical validation study on single cells, demonstrated that the NGS-based 24-aneuploidy screening protocol was accurate and reliable (Fiorentino *et al.*, 2014). The results provided 100% consistency for aneuploid embryo call with array-CGH, a well-established and highly validated method of aneuploidy screening.

The present study focused on the clinical potential of the NGS-based protocol for the detection of copy number changes of all chromosomes in embryos. A prospective trial involving analysis of human embryos at the blastocyst stage of development was designed to establish the concordance between the NGS copy number assignment with 24sure v3 array-CGH BAC-array.

Embryos obtained from 55 consecutive clinical PGS cycles, blindly assessed in parallel with both NGS and array-CGH techniques, displayed 100% concordance for transferable embryos. Consistency obtained during this investigation was similar to those of the previously published study that used NGS to examine single cell samples (Fiorentino *et al.*, 2014), demonstrating the equivalence to array CGH of the NGS-based method in the detection of chromosomal aneuploidy also in embryos at blastocyst stage derived from clinical PGS cycles.

From the clinical perspective, there has been increasing interest in screening blastocyst-stage embryos for chromosomal abnormalities, with a view to detecting and preferentially transferring euploid embryos during IVF cycles (Fragouli *et al.*, 2008, 2010; Schoolcraft *et al.*, 2010, 2011; Forman *et al.*, 2012). Biopsy at this stage has the advantage of allowing more cells to be sampled (∼5–10 cells), making comprehensive aneuploidy screening more robust (Schoolcraft *et al.*, 2010; Fiorentino, 2012). It also uses only trophectoderm cells, leaving the integrity of the inner cell mass, which goes on to form the fetus, intact.

However, as with cleavage-stage embryos, aneuploidy screening of embryos at blastocyst stage can be hampered by the presence of chromosomal mosaicism, which is a well-described phenomenon in the preimplantation embryo, characterized by the presence a mixture of diploid and aneuploid cell lines. Chromosomal mosaicism is relatively common in human blastocysts (van Echten-Arends *et al.*, 2011); therefore it is likely that the TE samples biopsied from a mosaic blastocyst include more than one cell line. As trophectoderm biopsy becomes the preferred stage for biopsy, mosaicism may represent an issue in the analysis and interpretation of the results after aneuploidy screening.

The NGS protocol for chromosomal analysis presented here has also detected atypical gains or/and losses that were below the automatic

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Table III Next-generation sequencing performance on blastocysts.

Concordance analysis	No. (95% CI)
Chromosome calling comparison	4545
Euploid chromosomes (true negatives)	4334
Aneuploid chromosomes (true positives)	211
Missed chromosome calls (false negatives)	0
Extra chromosome calls (false positives)	1
Aneuploidy call performance	
Sensitivity	100% (99.25–100%)
Specificity	99.98% (99.87–100%)
Whole-embryo aneuploidy/euploidy status comparison	
Euploid embryo (true negatives)	74
Aneuploid embryo (true positives)	106
Missed aneuploid embryo calls (false negatives)	0
Extra aneuploid embryo calls (false positives)	0
Aneuploid embryo call performance	
Sensitivity	100% (96.55–100%)
Specificity	100% (95.09–100%)
Positive predictive value	100% (96.55–100%)
Negative predictive value	100% (95.09–100%)

calling signal of the BFM Software. These findings could be interpreted both as artefacts of WGA or could be determined by the presence of chromosomal mosaicism. Twelve embryos, presenting atypical gains or/and losses only, were classified as with 'inconclusive' diagnosis (Table II); these embryos were cryopreserved and the results discussed with the patients in the course of a proper genetic counselling. Further investigations are ongoing in order to determine the actual chromosomal status of the embryos.

Another aspect of this study was the demonstration that the NGS-based 24-aneuploidy screening protocol has a high resolution and allows accurate detection of segmental imbalances as small as ∼14 Mb in size. The potential of identifying segmental changes has also been reported in earlier validation studies on single cells (Fiorentino et al., 2014) and is confirmed here on embryos at blastocyst stage.

As sequencing costs reduce further, allowing a greater read depth per sample for the same or reduced price, NGS approaches may also allow for simultaneous evaluation of single-gene disorders (Treff et al., 2013; Wells et al., 2013) and translocations (Yin et al., 2013) with comprehensive aneuploidy screening from the same biopsy without the need for multiple technological platforms.

Together, all these capabilities may provide a unique opportunity to reduce significantly the costs associated with PGS and PGD and thereby provide greater access to more patients. However, these predictions need to be validated by further studies with specific design objectives.

A further advantage related with the use of a Miseq sequencer is that the whole procedure can be completed in <24 h, a timeframe compatible with fresh embryo transfer.

Although there are many advantages related with the use of the NGS technology, the limitations must also be considered. Similar, to other technologies currently used for PGS, NGS cannot currently directly detect balanced chromosomal rearrangements, as there is no imbalance in the total DNA content. Moreover, although NGS has the potential to detect haploidy and some polyploidies using allele ratios, the sequences coverage and read depth of this protocol is insufficient to enable allele detection. It is also important to note the requirement of capital equipment expenses associated with the need for NGS instruments. Finally, potential cost benefits may not be achieved if there are insufficient samples available to fully utilize the available sequencing capacity in every run.

The clinical outcomes obtained in this study from PGS cycles performed with the NGS approach were very encouraging, resulting in a clinical pregnancy rate per embryo transfer of 63.8% (mean age 38.5 + 2.1 years) and an ongoing implantation rate of 62.0%. These values are comparable with recent results from other CCS approaches (Fragouli et al., 2010; Fiorentino et al., 2011; Forman et al., 2012; Scott et al., 2013). Although clinical results have documented high pregnancy rates following transfer of screened embryos, further data and broad based clinical application are required to better define the role of NGS in PGS applications. The only effective way to demonstrate its clinical effectiveness is to perform a well-designed and well-executed prospective randomized controlled trial, showing actual improvements in clinical outcomes. Such a trial, which represents the third phase of the study and will complete the initial validation of CCS using NGS technology, is forthcoming and will be conducted by involving a limited number of leading PGS laboratories. The results of the study will be critical when considering the use of this new technology in a clinical setting and will help to outline the potential for routine clinical use of NGS-based preimplantation embryo assessment of aneuploidy.

In conclusion, the results achieved in this study demonstrate the reliability of the NGS-based protocol for detection of whole chromosome aneuploidies and segmental changes in embryos. NGS methods may ultimately lead to reduced costs per patient, allowing IVF couples a wider use of PGS for choosing the most competent embryo(s) for transfer. NGS-based PGS represents a valuable alternative to other currently available CCS techniques, ready to find a place in routine clinical use in IVF.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

F.F. conceived the study, blindly assessed array-CGH and NGS results, performed data collection and data analysis and prepared the manuscript; S.B., A.B., A.N., E.C. and G.C. performed array-CGH/NGS experiments and blindly assessed the results; F.K. and C.-E.M. performed NGS data analysis, provided both guidance and technical support; M.G.M. performed the embryo laboratory procedures; E.G. was involved in patients' management and in critical discussion of the manuscript.

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Conflict of interest

Dr F.K. and C.-E.M. are full-time employees of Illumina, Inc. which provided NGS library and sequencing reagents for the study. All other authors have no conflicts to declare.

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