Abnormally fertilized oocytes can result in healthy live births: improved genetic technologies for preimplantation genetic testing can be used to rescue viable embryos in in vitro fertilization cycles

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Objective: To test whether abnormally fertilized oocyte (AFO)–derived blastocysts are diploid and can be rescued for clinical use.
Design: Longitudinal-cohort study from January 2015 to September 2016 involving IVF cycles with preimplantation genetic testing for aneuploidy (PGT-A). Ploidy assessment was incorporated whenever a blastocyst from a monopronuclear (1PN) or tripronuclear zygote (2PN + 1 smaller PN; 2.1 PN) was obtained.
Setting: Private IVF clinics and genetics laboratories.
Patient(s): A total of 556 women undergoing 719 PGT-A cycles.
Intervention(s): Conventional chromosome analysis was performed on trophectoderm biopsies by quantitative polymerase chain reaction. For AFO-derived blastocysts, ploidy assessment was performed on the same biopsy with the use of allele ratios for hetorozygous SNPs analyzed by means of next-generation sequencing (1:1 = diploid; 2:1 = triploid; loss of heterozygosity = haploid). Balanced-diploid 1PN- and 2.1PN-derived blastocysts were transferred in the absence of normally fertilized transferable embryos.
Main Outcome Measure(s): Ploidy constitution and clinical value of AFO-derived blastocysts in IVF PGT-A cycles.
Result(s): Of the 5,026 metaphase II oocytes injected, 5.2% and 0.7% showed 1PN and 2.1PN, respectively. AFOs showed compromised embryo development ($P<.01$). Twenty-seven AFO-derived blastocysts were analyzed for ploidy constitution. The 1PN-derived blastocysts were mostly diploid (n = 9/13; 69.2%), a few were haploid (n = 3/13; 23.1%), and one was triploid (n = 1/13; 7.7%). The 2.1PN-derived blastocysts were also mostly diploid (n = 12/14; 85.7%), and the remainder were triploid. Twenty-six PGT-A cycles resulted in one or more AFO-derived blastocysts (n = 26/719; 3.6%). Overall, eight additional balanced-diploid transferable embryos were obtained from AFOs. In three cycles, the only balanced-diploid blastocyst produced was from an AFO (n = 3/719; 0.4%). Three AFO-derived live births were achieved: one from a 1PN zygote and two from 2.1PN zygotes.
Conclusion(s): Enhanced PGT-A technologies incorporating reliable ploidy assessment provide an effective tool to rescue AFO-derived blastocysts for clinical use. (Fertil Steril® 2017;108:1007–15. ©2017 by American Society for Reproductive Medicine.)
Key Words: Ploidy, preimplantation genetic testing, PGT

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Received June 7, 2017; revised July 21, 2017; accepted August 7, 2017; published online September 15, 2017.
A.C. has nothing to disclose. N.T. has nothing to disclose. D.C. has nothing to disclose. X.T. has nothing to disclose. S.F. has nothing to disclose. A.V. has nothing to disclose. S.C. has nothing to disclose. R.M. has nothing to disclose. G.O. has nothing to disclose. C.S. has nothing to disclose. R.S. has nothing to disclose. F.M.U. has nothing to disclose. L.R. has nothing to disclose.
Supported by Genera, Genetyx, and Reproductive Medicine Associates of New Jersey.
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Fertility and Sterility® Vol. 108, No. 6, December 2017 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2017.08.004

One of the main rate-limiting steps in in vitro fertilization (IVF) is the availability of viable embryos to be used for transfer in each cycle. This is particularly relevant for patients with poor prognosis due to advanced female age or showing a poor response to controlled ovarian stimulation. In these couples, the limited number of oocytes results in a significant reduction of the chance to achieve a live birth (1).

After oocyte retrieval, insemination is performed with the use of conventional IVF or intracytoplasmic sperm injection (ICSI). In all IVF laboratories, a fertilization check is performed 16–18 hours after insemination and requires the confirmation of the extrusion of the second polar body and determination of the number and shape of the pronuclei (PN) (2, 3). Those zygotes displaying successful extrusion of the second polar body and two even PN are considered to be "normally fertilized" and are cultured further to monitor embryo quality and development. In contrast, those zygotes showing a single or more than two PN are considered to be "abnormally fertilized." Even though the embryos deriving from abnormally fertilized oocytes (AFOs) are capable of normal in vitro development, they are usually discarded because of a higher risk for abnormal ploidy constitution (i.e., haploidy, triploidy, or tetraploidy) (3).

Zygotes with one PN are thought to be at a higher risk of being haploid, and the transfer of these embryos is expected to result in an implantation failure. However, the origin of a monopronucleated zygote after ICSI could be parthenogenetic oocyte activation or an abnormal formation of the nuclear envelope. The latter may result either from the combination of the two genomes into a single PN or from the failure to organize a nuclear envelope around one of the parental genomes. Recently, several articles about the possible origins of zygotes with one PN and the chromosomal constitution of the resulting embryos have been published (4–6). From these reports, it can be assumed that a considerable number of embryos originating from zygotes with one PN could have a normal chromosomal constitution, and as a result they could be considered for reproductive purposes in cases where no embryos deriving from normally fertilized zygotes are available (7–9).

Embryos arising from zygotes with three PN are considered to harbor a polyploid chromosomal constitution, and the transfer of these embryos is expected to result in a higher risk for miscarriage and molar pregnancy with an associated 2.5% risk of developing into choriocarcinoma (10). However, no definitive genetic evidence has been obtained showing that all resulting zygotes are chromosomally abnormal. It is possible that some may be chromosomally normal despite the morphologic defects (10).

Overall, ~10% of inseminated oocytes fertilize abnormally, and the embryos deriving from them are typically discarded in the absence of a reliable approach to monitor their genetic risk for ploidy defects. Indeed, even when aneuploidy testing is performed with the use of conventional comprehensive chromosome testing (CCT), owing to the normalization method ploidy analysis is not possible and embryos showing a normal chromosomal profile can still be tetraploid, triploid, or haploid. In the present study, we report the development, validation, and clinical application of an enhanced CCT protocol for the detection of aneuploidies and parallel ploidy assessment.

After being validated in the preclinical phase, the combined preimplantation genetic testing (PGT) scheme was implemented clinically in IVF cycles undertaken by poor-prognosis patients, with the aim of rescuing the embryos deriving from AFOs, which would have otherwise been discarded.

MATERIALS AND METHODS

Study Design

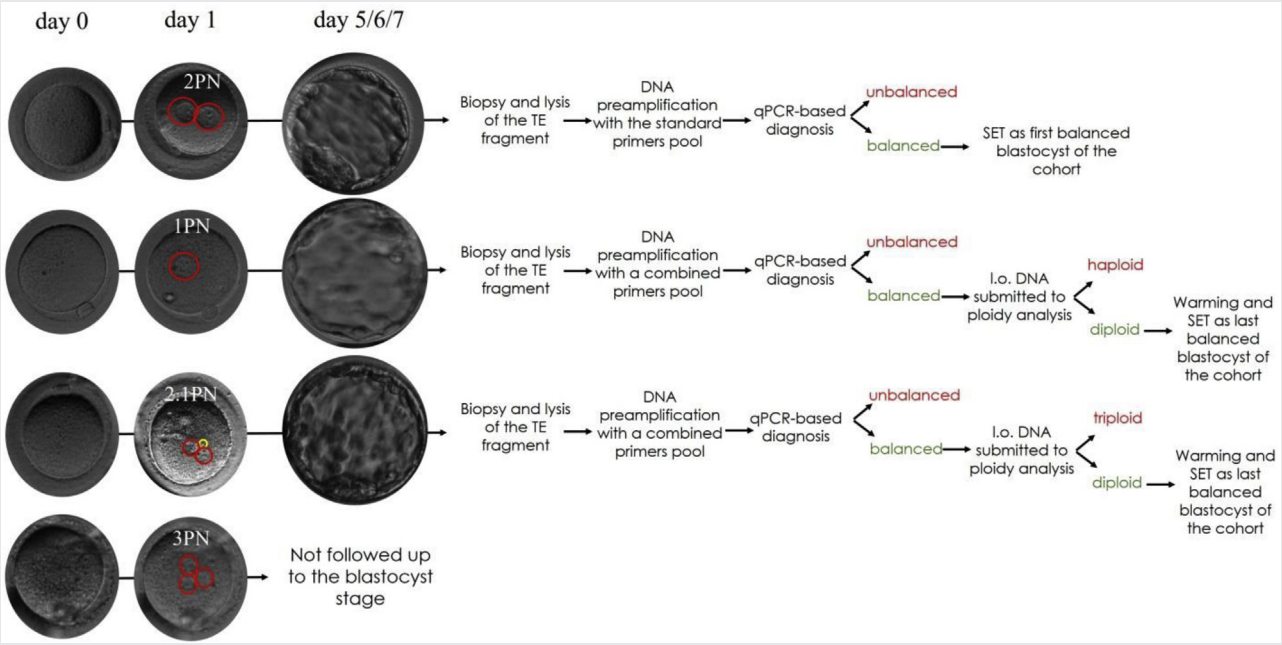
This is a longitudinal cohort study performed from January 2015 to September 2016 involving 678 consecutive patients undergoing preimplantation genetic testing for aneuploidies (PGT-A) cycles at Genera IVF center in Rome. PGT-A was offered to patients of advanced female age (>35 y) or to younger patients with a history of unsuccessful IVF treatments (more than two failed IVF cycles) or previous spontaneous abortion (more than two miscarriages).

The preclinical validation of the protocol for the combined aneuploidy and ploidy assessment from a single sample was carried out on a set of cell lines with previously established triploid karyotypes (Coriell Cell Repository IDs AG05025, AG06266, GM10013, GM01672, GM04376, and GM04939). Six-cell aliquots of diploid cell lines and triploid cell lines were processed to mimic the approximate number of cells in a trophectoderm biopsy. All samples were processed blindly.

From January 2015, the combined aneuploidy and ploidy analysis was systematically offered to all consenting patients undergoing PGT-A when a blastocyst from a monopronuclear (1PN) or tripronuclear (two evenly sized PN plus one smaller PN; 2.1PN) zygote was obtained (Fig. 1). To start with a more gradual and conservative approach, we decided to analyze only tripronuclear zygotes showing one smaller supernumerary PN, excluding from clinical use the tripronuclear (3PN) zygotes showing three evenly sized PNs. In our standard practice, 2.1PN are still considered to be unsuitable for clinical use owing to similar concerns of ploidy alterations. A specific consent form was provided and submitted to patients undergoing IVF before ovarian stimulation was started. The counseling involved a discussion of the genetic risks associated with the use of AFOs, their clinical fate when no testing is performed, and the possibility to have them rescued for clinical use by means of an improved PGT-A analysis. The limitations of this experimental protocol were also clearly detailed. No extra costs were associated with this additional genetic procedure.

As a general policy, balanced (without chromosome copy number alteration) embryos from 2PN zygotes were always selected first for transfer. Balanced-diploid embryos obtained from 1PN or 2.1PN zygotes were considered to be suitable only in the absence of balanced normally fertilized embryos (Fig. 1). The infertility treatment protocols, including hormonal stimulation, oocyte retrieval, IVF, embryo culture, embryo morphologic evaluation, biopsy, transfer methods, and clinical outcomes assessment applied in this study have

FIGURE 1



Clinical study design. In all consecutive cycles with preimplantation genetic testing for aneuploidies (PGT-A), the fertilization check was performed on day 1 (16–18 hours) after insemination or in a time-lapse system. Normally fertilized oocytes showing two evenly sized pronuclei (PN) were grown to the blastocyst stage and submitted to quantitative polymerase chain reaction (qPCR)–based aneuploidy testing as a standard procedure. The abnormally fertilized oocytes (AFOs) showing three evenly sized PN (3PN) were not followed further. The AFOs showing either 1PN or 2.1PN, the latter defined as the presence of 2 well defined PN (outlined in red in the figure) and an additional smaller PN (not larger than one-third the size of normal; outlined in yellow in the figure), were kept in culture up to the blastocyst stage. If they developed as blastocysts, they underwent trophectoderm (TE) biopsy and qPCR-based chromosome copy number analysis (first-line PGT-A assessment). In embryos diagnosed as balanced for chromosome copy number, the left-over (l.o.) embryonic DNA was submitted for additional ploidy analysis. In the absence of any other normally fertilized–derived transferable blastocyst, AFO-derived balanced-diploid embryos were warmed and transferred in a single-embryo transfer (SET) policy.

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been previously described (11–13). The Institutional Review Board of the Clinica Valle Giulia approved the study.

Fertilization Check

All metaphase II (MII) oocytes were inseminated by means of ICSI 36–38 hours after hCG administration with the use of a previously described technique and instrumentation (14). Injected oocytes were individually cultured in standard benchtop incubators (MINC benchtop incubator; Cook Medical; n = 2,802 zygotes included in the study: 149 1PN, 20 2.1PN, and 2,633 2PN) or time lapse monitoring (TLM) systems (Embryoscope [Unisense] or GERI [Genea]; n = 983 zygotes included in the study: 51 1PN, 7 2.1PN, and 925 2PN), depending on the daily availability of the instrumentations and without any specific selection criteria. For standard culture, fertilization was assessed 16–18 hours after injection. Those zygotes displaying 2PN and a second PB were considered normally fertilized and cultured further. Monopronucleated zygotes (1PN) were defined whenever a single PN was present and, for those cultured in standard incubators, a reevaluation within 4 hours from the first check was performed to confirm the absence of a second PN. 2PN zygotes showing the appearance of a second PN at the second observation (n = 2 out of 2/802 zygotes [0.07%]; n = 2 out of 151 zygotes [1.3%] showing a single PN at the first observation) were considered to be normally fertilized in this study. The zygotes showing two evenly sized PN and one extra smaller PN were defined as 2.1 PN (Fig. 1) and cultured further to the blastocyst stage for chromosomal analysis. Zygotes showing three evenly sized PN or more were not cultured further and were discarded in this preliminary clinical application.

For TLM-based monitoring, continuous sequential observations allowed the exclusion of sporadic cases of asynchronous 2PN formation or 2PN fusion (n = 4 out of 983 zygotes [0.4%]; n = 4 out of 55 zygotes [7.3%] that would have shown a single PN at a single conventional fertilization check), thus providing a more objective evaluation.

First-Line Chromosomal Analysis

All trophectoderm biopsies were processed for quantitative polymerase chain reaction (qPCR)–based whole chromosome copy number analysis at Genetyx as previously described (15). Briefly, multiplex amplification of 96 loci (four per chromosome) was carried out, and a method of relative

quantification (16) was applied to predict the copy number status of each chromosome (Supplemental Fig. 1, available online at www.fertstert.org). This methodology was designed to specifically identify only whole chromosome (not segmental) aneuploidies and was validated in preclinical (15) and clinical studies (17) at Reproductive Medicine Associates of New Jersey (RMA-NJ) as well as at the Genetyx molecular genetics laboratory (18). A karyotype prediction was made for each embryo by a certified cytogeneticist.

Next-Generation Sequencing–Based Ploidy Assessment

40–Single-nucleotide polymorphism method. Whenever a blastocyst was obtained from an AFO, an independent set of primers for 40 highly variable single-nucleotide polymorphisms (SNPs) was incorporated in the preamplification reaction (19), and a 25-μL aliquot of the preamplification product was submitted for next-generation sequencing (NGS)–based quantification of allelic ratios at RMA-NJ (Fig. 1). Re-preamplification was set up with 25 μL of the remaining preamplification product, 50 μL 2× Taqman Preamplification Master Mix (Thermo Fisher Scientific), and 25 μL primer pool of 40-SNP TaqMan genotyping assays (0.2×) in 100-μL reactions. PCR cycling conditions were set to 10 minutes at 95°C followed by 30 cycles of 15 seconds at 95°C and 4 minutes at 60°C in an Applied Biosystems 2720 thermocycler (Thermo Fisher Scientific). One μL of re-preamplified DNA was assessed with the use of D1k Screentape (Agilent Technologies) and then was normalized to 200 ng in a total volume of 35 μL molecular biology–grade water. Ion Xpress Plus Fragment Library Kit and Ion Xpress Barcode Adapters 1–96 Kit were used to construct the whole-genome amplification library as recommended by the supplier (Thermo Fisher Scientific). One μL of amplified library was assessed with the use of D1k Screentape. Individual libraries were diluted to 100 pmol/L with low–Tris-EDTA buffer (Thermo Fisher Scientific). An equal amount of each of 12 samples was pooled together for one Ion PI Chip V3 (Thermo Fisher Scientific). Ion Sphere particles containing clonally amplified DNA were prepared with the use of Ion PI Template OT2 200 Kit v3, and the template-positive Ion Sphere particles were then enriched with the use of Ion Onetouch ES (Thermo Fisher Scientific). The enriched template-positive Ion Sphere particles were sequenced with the use of Ion PI Chip v3 and Ion PI Sequencing 200 Kit v3 on the Ion Proton instrument (Thermo Fisher Scientific). The sequencing reads were aligned to the sequence around the targeted SNPs, and the genotype was called with torrent variant caller 5.0. The SNPs with a depth <100 were filtered out. The allele ratios from heterozygous SNPs were used to assess embryo ploidy based on the deviation from the normal 1:1 ratio. Loss of heterozygosity (LOH) is indicative of a haploid complement, whereas a 2:1 ratio is indicative of triploidy. The distribution of heterozygous allele ratios was compared between diploid and triploid cell samples to predict the ploidy status and set thresholds for discrimination as well as demonstrate validity of the methodology (Supplemental Fig. 1).

For haploid embryos, no heterozygous SNPs would be expected, even though the SNP analysis in this case is not able to discriminate between true haploid cells (a single set of 23 chromosomes) and diploid cells with two identical genome sets that might theoretically originate in extremely rare cases following diploidization of an haploid conception. However, haploidy has been shown to persist until the blastocyst stage in both murine and human parthenogenetic embryos, and diploid cells progressively dominate over increasing cell cycles owing to spontaneous diploidization only at later stages after parthenogenetic embryonic stem cell derivation, well after the blastocyst stage (20, 21). Thus, the haploid configuration (a single set of 23 chromosomes) is the most likely when observing a complete loss of hetorozygosity in blastocyst biopsies. Moreover, both configurations are not viable and can be considered to be equivalent in terms of embryonic reproductive potential.

Targeted-NGS method. A direct targeted-NGS approach was used for confirmation of ploidy on blastocyst rebiopsies. Amplification for targeted NGS was performed with the use of primers that included adapter A (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3′) in the forward primer and adapter trP1 (5′-CCTCTCTATGGGCAGTCGGTGAT-3′) in the reverse primer. Sequencing was performed with the use of an Ion PI Chip v2 for NGS on the Ion Proton System according to manufacturer specifications (Thermo Fisher Scientific). Amplicons were specifically designed to contain highly variable SNPs for both autosomes and sex chromosomes. Allele ratios were calculated with the use of heterozygous SNPs (Supplemental Fig. 1). All of the autosomal SNPs with a heterozygous call (from the 2,571 SNPs genotyped) were selected for each sample, and a specific average allele ratio was calculated. Because diploid specimens have two copies of each autosome, a 1:1 ratio is expected between the major and minor allele frequencies for a given heterozygous SNP. In contrast, if three copies of all chromosomes are present, then a 2:1 ratio is expected, because all of the preselected SNPs only had two alternate alleles. Genome-wide LOH was used to confirm the haploid constitution.

Outcome Measures

The genetic classification of embryos after the integrated genetic scheme was as follows. Balanced embryos were defined as those with the suffix male (presence of the Y chromosome) or female (absence of the Y chromosome) when a normal result was obtained after the 24-chromosome qPCR-based first-line genetic analysis. Unbalanced embryos were defined as those where an extra or a missing chromosome detected. After the second-line ploidy analysis, embryos were classified as follows: balanced-diploid, balanced-haploid, or balanced-triploid whenever a diploid, haploid, or triploid constitution without individual chromosome aneuploidy was revealed (Supplemental Fig. 1) and unbalanced-diploid, unbalanced-haploid, or unbalanced-triploid whenever a diploid, haploid, or triploid constitution and an extra or a missing chromosome was observed.

Clinical outcomes of the PGT-A cycles were defined as follows: ongoing pregnancy as the presence of a fetus with

heart activity beyond 20 weeks of gestation and live birth as the delivery of a baby, following the IMPRINT (Improving the Reporting of Clinical Trials of Infertility Treatments) guidelines (22). The 6-month cumulative ongoing pregnancy rate was defined as the number of ongoing pregnancies or live births divided by the total number of patients enrolled in the study within 6 months after oocyte retrieval.

Statistical Analysis

Continuous data are presented as mean ± SD with range and 95% confidence interval (CI). Categoric variables are presented as n (%) and 95% CI. Fisher exact test and analysis of variance with Bonferroni correction were used to assess differences between categoric and continuous variables, respectively. Alpha was set at 0.05 for single comparisons and to 0.002 when Bonferroni correction for multiplicity of testing was applied. All analyses were carried out with the use of the statistical software R version 2.14.2 (Free Software Foundation).

RESULTS

Validation of the NGS-Based Protocol for the Detection of Ploidy State After Conventional qPCR-Based CCT

To validate the protocol, a blinded analysis of 33 6-cell aliquots of previously established triploid (GM04939 = 6; AG05025 = 5) and diploid (GM0323 = 12; GM4305 = 11) cell lines was carried out. All of the results predicted the correct karyotype for all samples. Allelic ratios of 2.12 ± 0.12 were observed for triploid samples (range 1.97–2.31, 95% CI 1.99–2.24). Diploid samples showed a mean allelic ratio of 1.29 ± 0.10 (range 1.18–1.50, 95% CI 1.22–1.36).

Embryologic Data

In the study period, 5,026 MII oocytes were injected and 75.3% (n = 3,785/5,026; 95% CI 74.1%–76.5%) were included in the study (Supplemental Table 1, available online at www.fertstert.org). Embryo development was significantly compromised in 1PN and 2.1PN embryos. Indeed, they showed a significantly higher probability of developmental arrest at the 1–2-cell division stage (Fig. 2). As many as 60% (n = 120/200; 95% CI 52.8%–66.8%) and 25.9% (n = 7/27; 95% CI 11.1%–46.2%) of 1PN and 2.1PN zygotes, respectively, failed to cleave compared with only 1.8% of matched sibling normally fertilized zygotes (n = 66/3558; 95% CI 1.5%–2.4%; $P<.01$). Cleaved 1PN and 2.1PN embryos progressed at rates similar to matched control embryos (Fig. 2). However, the final blastocyst formation rate was significantly reduced only for 1PN zygotes compared with both matched and overall normally fertilized control zygotes (Fig. 2; Supplemental Table 1; $P<.01$). The day of blastocyst formation and blastocyst morphology was similar between abnormally and normally fertilized embryos, although more data are required from future studies (Supplemental Table 1). In the study period, 27 expanded blastocysts were obtained from AFOs, with a similar contribution from 1PN (n = 13) and 2.1PN (n = 14) zygotes. More 1PNs were obtained after ICSI cycles, but they had lower blastocyst formation than 2.1PNs (Fig. 2; Supplemental Table 1; $P<.01$).

All of these embryos underwent trophectoderm biopsy, qPCR-based PGT-A, and second-line ploidy assessment.

Genetic Data

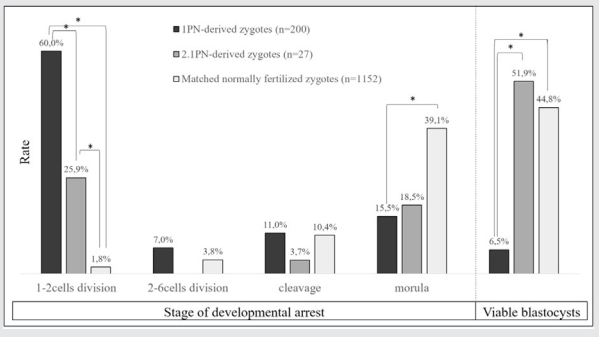
The first-line chromosomal analysis was performed by assessing whole-chromosome aneuploidies for all autosomes and sex chromosomes. All AFO-derived embryos were also evaluated for their ploidy constitution by means of the NGS-based analysis of 40 highly polymorphic SNPs whenever a balanced chromosomal constitution was observed at the first-line genetic analysis (Fig. 1; Table 1; Supplemental Fig. 1)

For validation purposes, a blinded assessment on a second trophectoderm biopsy was performed by means of the targeted-NGS approach for ten unbalanced blastocysts that were not originally evaluated for ploidy constitution and for eight balanced blastocysts to confirm both the chromosomal and ploidy diagnoses. Specifically, the blinded ploidy evaluation was performed by interrogating an average of 2,400 SNPs across the genome (Table 1; Supplemental Fig. 1).

As presented in Table 1 and Figure 3, most of the 1PN blastocysts followed for ploidy complement were diploid (n = 9/13; 69.2%, 95% CI 38.6%–90.9%), and three were haploid (n = 3/13; 23.1%, 95% CI 5.0%–53.8%). Remarkably, one blastocyst obtained from a 1PN zygote resulted in a diagnosis of triploidy (n = 7.7%; 95% CI 0.2%–36.0%; Table 1; Fig. 3). Most of the 2.1PN embryos also showed a diploid configuration (n = 12/14; 85.7%, 95% CI 57.2%–98.2%), and the remainder were triploid (n = 2/14; 14.3%, 95% CI 17.8%–42.8%; Table 1; Fig. 3). All of the reanalyses of embryos with a previously established ploidy gave concordant results (Table 1).

The evaluation of the Y and X chromosome copy number values in relation to the autosomes could be used to predict a triploid XXY constitution based on the first qPCR analysis.

FIGURE 2



Embryo developmental arrest per stage of preimplantation development and viable blastocyst rates for 1PN, 2.1PN, and matched normally fertilized zygotes. Only the matched normally fertilized zygotes from the 181 PGT-A cycles where at least one 1PN- and/or 2.1PN-derived zygote was obtained were used for comparison of viable blastocyst rates. PN = Pronuclei. *Statistically significant difference.

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TABLE 1

Embryologic, genetic, and clinical data of the blastocysts derived from abnormally fertilized oocytes.

Sample ID	Female age (y)	Indication for PGT-A	PGT-A cycle	Total no. of 2PN blastocysts biopsied (balanced)	Fertilization abnormality	Blastocyst morphology	Day of biopsy	qPCR diagnosis	Ploidy analysis (40 SNPs)	Ploidy analysis (targeted NGS)	Culture system	Clinical outcome
10343_7[a]	38	AMA	2	3 (1)	2.1PN	C11	5	Balanced female	Diploid	–	S	Delivery
11680_1[a]	38	AMA	1	6 (4)	2.1PN	C11	6	Balanced female	Diploid	–	S	/
11793_8[a]	42	AMA	1	5 (0)	2.1PN	C22	6	Balanced female	Diploid	–	S	Delivery
13037_2[a]	35	AMA	1	3 (1)	2.1PN	C33	7	Balanced male	Diploid	–	TLM	Implantation failure
13675_6.1[a]	42	AMA	1	8 (1)	2.1PN	B11	6	Balanced male	Diploid	–	TLM	–
13421_2bis[a]	42	AMA, RIF	1 (LPS)	0	1PN	B11	6	Balanced male	Diploid	Diploid	S	Delivery
13264_6[a]	34	RIF	1	2 (2)	1PN	C22	6	Balanced female	Diploid	–	TLM	Implantation failure
13814_4.3[a]	40	AMA	2	3 (0)	1PN	C11	6	Balanced female	Diploid	–	S	–
11680_7	38	AMA	1	6 (4)	2.1PN	C12	6	Balanced female	Triploid	Triploid	S	
13379_9	39	AMA	1	5 (2)	2.1PN	C22	5	Unbalanced, XXY	Triploid	Triploid	S	
9949_5	41	AMA	2	1 (1)	2.1PN	C33	7	Unbalanced male, −1	NA	Diploid	S	
12673_4	43	AMA	2	3 (0)	2.1PN	C33	6	Unbalanced male, +15, −21	ND	Diploid	S	
14461_2.2	43	AMA	1	1 (0)	2.1PN	C33	6	Unbalanced female, −16	Diploid	–	S	
9414_1bis	39	AMA	1 (LPS)	3 (3)	2.1PN	C11	5	Unbalanced female, +15, +18	–	Diploid	S	
11795_8	39	AMA	2	2 (2)	2.1PN	C33	7	Unbalanced male, + 7	–	Diploid	S	
12830_9	41	AMA	1	9 (3)	2.1PN	C33	7	Unbalanced male, −4,-16	–	Diploid	TLM	
13540_3	44	AMA	1 (FPS)	0	2.1PN	C33	6	Unbalanced male, −15, +16	–	Diploid	S	
13184_1	36	AMA	1	4 (4)	1PN	C11	5	Unbalanced male, +13	Diploid	ND	S	
9924_1	36	AMA, RIF, RPL	4	3 (0)	1PN	C11	6	Balanced female	Haploid	Haploid	S	
5100_6	36	AMA	1	3 (2)	1PN	C11	5	Unbalanced female, +15	Diploid	Diploid	TLM	
12713_12bis	38	AMA	1 (LPS)	6 (3)	1PN	C33	6	Unbalanced female, +8	Diploid	Diploid	TLM	
12684_8	34	RIF	1	7 (4)	1PN	C22	7	Balanced female	Haploid	Haploid	S	
13076_3.7	34	RIF	1	3 (2)	1PN	C11	5	Balanced female	Haploid	Haploid	TLM	
11488_4	44	AMA	1	1 (0)	1PN	C22	6	Unbalanced male, +15, +20, −21	–	Diploid	S	
9321_5	35	AMA, RIF	1	7 (6)	1PN	C22	6	Unbalanced female, −X	–	Diploid	S	
12884_2	38	AMA	1	1 (0)	1PN	D11	6	Unbalanced female, +22	–	Triploid	S	
13591_9	42	AMA	1	3 (0)	1PN	C33	6	Unbalanced female, −18	–	Diploid	TLM	

Note: AMA, advanced maternal age; FPS, follicular phase stimulation; LPS, luteal phase stimulation; NA, not amplified; ND, not determined; NGS, next-generation sequencing; PN, pronuclei; PGT-A, preimplantation genetic testing for aneuploidies; RIF, recurrent implantation failure; RPL, recurrent pregnancy loss; S, standard; SNPs, single-nucleotide polymorphisms; TLM, time-lapse microscopy.

[a] Transferable blastocysts.

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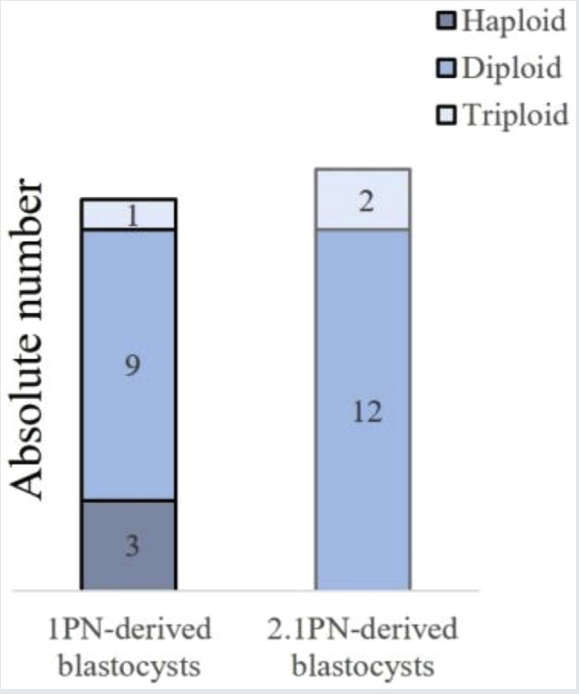
Indeed, in these cases the ratio of chromosome copy number to the autosomes is expected to be 0.66 and 0.33 for the X and Y chromosomes, respectively, as shown in Supplemental Figure 2 (available online at www.fertstert.org). Furthermore, in this series of cases, all of the 1PN embryos showing a Y chromosome at the first-line genetic analysis were reported to be diploid at the ploidy follow-up analysis (Table 1). In this regard, even though more data are required, it might be presumed that the first-line genetic analysis provides a considerable predictive value of the ploidy status whenever a Y chromosome is detected. For female embryos, instead, the specific second-line ploidy analysis is critical.

In the study period, six blastocysts were obtained from early-cleaving zygotes (first mitotic division occurred <21 hours after injection) cultured in a static system where, at the fertilization check (16–18 hours after injection), the PN had already disappeared (0PN2CP and 0PN1CP; Supplemental Table 2, available online at www.fertstert.org). Trophectoderm biopsy–based genetic analysis of aneuploidies and ploidy constitution was here adopted to rule out a putative haploid/polyploid chromosomal complement. A diploid constitution was observed for all six embryos, suggesting that the ploidy assessment in these cases can be used as an alternate approach to ensure the transfer of diploid embryos when PN have already disappeared at the fertilization check.

Furthermore, a proof-of-concept analysis was conducted for zygotes showing 3PN (Supplemental Table 2) to investigate their ploidy complement. These zygotes represent ~5% of fertilized zygotes after ICSI (n = 203/4104; 5.0%, 95% CI 4.3%–5.7%; Supplemental Table 1) and are capable of normal development to the blastocyst stage. The analysis of four 3PN-derived blastocycsts revealed a diploid constitution for two of them. These data paved the way to the inclusion of 3PN-derived blastocysts for clinical use in IVF, provided that the monitoring of the ploidy constitution could be offered.

FIGURE 3



Overall results of ploidy analysis in blastocysts derived from either 1PN or 2.1PN zygotes. PN = pronuclei.

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Clinical Data

In the study period, 678 patients undergoing a PGT-A cycle were given the option of having their AFO-derived blastocysts included for ploidy assessment. The clinical workflow is detailed in Supplemental Figure 3 (available online at www.fertstert.org). Of these, 556 (81.9%; Supplemental Fig. 3; Supplemental Table 3, available online at www.fertstert.org) consented to participate to the study. The characteristics of the patients included in this analysis were typical of a poor-prognosis population, as already suggested by the advanced mean maternal age (39.2 ± 3.2 y, range 27–44 y; Supplemental Table 3). Overall, 227 AFOs were obtained in 719 PGT-A cycles, with a mean number of 0.3 ± 0.7 (range 1–5) per cycle (Supplemental Fig. 3). In 181 out of 719 PGT-A cycles (25.2%, 95% CI 22.0%–28.5%), at least one AFO was obtained that was further cultured to the blastocyst stage for trophectoderm biopsy and chromosome diagnosis (Supplemental Fig. 3).

On a per–oocyte retrieval basis, 26 PGT-A cycles resulted in at least one blastocyst obtained from AFOs (3.6%, 95% CI 2.4%–5.2%; Supplemental Fig. 3). In three PGT-A cycles the only blastocyst(s) produced derived from AFOs (0.4%, 95% CI 0.09%–1.2%; Supplemental Fig. 3). Overall, the clinical use of AFOs resulted in eight additional transferable balanced-diploid blastocysts from six PGT-A cycles (0.8%, 95% CI 0.3%–1.8%; Table 1; Supplemental Fig. 3).

Two 1PN-derived balanced-diploid blastocysts were transferred, and one resulted in a live birth (Table 1). Three 2.1PN-derived balanced-diploid blastocysts were transferred, and two resulted in live births (Table 1). The remaining three balanced-diploid AFO-derived blastocysts had not been transferred yet at the time of writing. All babies showed normal obstetrical and perinatal outcomes.

The 6-month cumulative live birth rate (CLBR) per oocyte retrieval increased from 17.2% (n = 124/719; 95% CI 14.6%–20.2%) to 17.7% (n = 127/719; 95% CI 14.9%–20.7%) with the inclusion of AFOs for clinical use in this cohort of patients.

DISCUSSION

Eukaryotic organisms usually contain a diploid complement of chromosomes. The diploid state seems to be preferred because it enables sexual reproduction and facilitates genetic recombination. In humans, genetic variations that alter the ploidy state are not compatible with life, with triploid conception being at risk for clinical miscarriages and molar pregnancies, whereas haploids are expected to result in implantation failure. Embryos bearing an abnormal ploidy configuration usually originate from digynic or dispermic conception or parthenogenic activation, showing an abnormal number and shape of PN at the fertilization check in IVF treatments. Accordingly, in IVF practice worldwide, AFOs are routinely

discarded owing to the increased risk and concerns for ploidy abnormalities. Generally, this loss accounts for 10% of the zygotes obtained.

Different studies have previously shown that a considerable proportion of 1PN and 3PN zygotes are diploid (4, 10, 23, 24), and some reports already highlight their potential use for reproductive purposes. In recent publications, several healthy babies born from 1PN fertilized oocytes have been reported (7–9). However, none of these studies provided a genetic analysis to assess the embryonic ploidy before embryo transfer, and only 1PN-derived embryos have been selected for clinical use owing to the increased risk for polyploidy when dealing with 3PN-derived embryos. In the present study, we report the first systematic clinical application of an enhanced genetic approach aimed at rescuing AFOs during PGT-A cycles by means of the direct assessment of their ploidy.

Remarkably, most of the AFOs analyzed in this clinical series of cases showed a diploid chromosomal complement. Of the 27 blastocysts obtained from 1PN and 2.1PN zygotes during IVF cycles, 77.8% (n = 21) were diploid. Surprisingly, 1PN-derived blastocysts were found to bear all possible ploidy configurations, even triploid in one case. From a basic biology perspective, haploid blastocysts of excellent morphology were also observed (n = 7/13; 53.8%; Supplemental Table 1), suggesting that haploinsufficiency for the entire genome is still compatible with embryo preimplantation progression and apparently normal inner cell mass and trophectoderm differentiation. These data are consistent with recent findings in the mouse model showing that the depletion of genes (null mutant alleles) involved in the establishment and maintenance of blastomere polarity does not seem to compromise preimplantation development and morphology (25–30). The finding that 1PN-derived blastocysts can also be triploid suggests that additional caution is required when considering the clinical use of genetically untested 1PN embryos for reproductive purposes without the monitoring of their ploidy constitution, as recently reported by Itoi et al. (7) and Bradley et al. (9). In particular, Bradley et al. analyzed short tandem-repeat (STR) loci to detect maternal and paternal DNA contributions to the embryos in 1PN blastocysts to complement the standard array comparative genome hybridization or NGS PGT-A scheme. In this way, they have been able to confirm the biparental contribution to the embryo, excluding the monoparental inheritance, but no direct evidence about the ploidy constitution could be obtained (9). As shown in our study, the direct analysis of the ploidy constitution of 1PN blastocysts revealed that they can also be triploid. These cases will go undetected if the genetic analysis relies solely on STR analysis. We therefore reccomand the clinical use of 1PN blastocysts only after a direct validated ploidy assessment.

In this preliminary clinical application, six patients (0.8%) had one balanced-diploid embryo deriving from the incorporation of AFOs in the PGT-A program that would have otherwise been discarded. Notably, in three cycles the only balanced-diploid embryos were derived from AFOs. Five balanced-diploid embryos from AFOs were transferred and the first three live births were obtained from this cohort of patients, and a further three were still cryopreserved at the time of writing. One of the three live births was obtained from a 1PN-derived embryo, which was the only blastocyst obtained after follicular phase and luteal phase stimulations and oocyte retrievals within the same menstrual cycle (11). According to a conservative estimate of these data, it may be concluded that, in this poor-prognosis patient population, one additional live birth could be obtained per 185 oocyte retrievals thanks to the incorporation of 1PN and 2.1PN zygotes for clinical use. However, despite the objective improvement in the CLBR achievable, the reproductive potential of diploid embryos obtained from AFOs could not be powerfully ascertained in the present study owing to the low number of replacements performed. Future studies are needed to properly address this outcome.

These data pave the way for a new application of PGT-A as an objective approach to rescue viable embryos derived from AFOs which are normally discarded from clinical use. Implementation of effective embryo cryopreservation approaches and single-embryo transfer policies has sharply increased over the past 10–15 years (31). Outcomes per fresh embryo transfer are no longer meaningful to patients and clinicians when attempting to estimate the chance of achieving a live birth in IVF (32–34). Indeed, it has been suggested that the most appropriate parameter to report IVF outcomes should be the CLBR per started ovarian stimulation or oocyte retrieval, thus including all fresh and subsequent frozen embryo transfers (35–37). The ultimate information that patients, providers, and policy makers require is the chance of delivering a healthy baby per started treatment (38, 39) or per defined period, and improvements in clinical practice toward this end are desirable. Conventionally, PGT-A is intended as an embryo selection approach that may improve IVF outcomes from a per-transfer perspective by increasing the application of the elective single-embryo transfer policy and reducing the incidence of miscarriages and chromosomally abnormal pregnancies but with no effect on the CLBR (11). As shown in the present study, the enhancement and evolution of the genetic technologies adopted for PGT-A provide an effective strategy to increase both the safety of the treatment, through the selection of chromosomally balanced embryos for transfer, and moderately the CLBR per oocyte retrieval, by rescuing AFOs for clinical use which would otherwise be discarded for their increased risk for ploidy abnormalities. Moreover, with the possible inclusion of 3PN blastocysts for chromosome analysis and transfer, in case of a balanced-diploid constitution, a further increase of CLBR in PGT-A cycles is foreseeable.

Remarkably, the implementation of this new PGT-A scheme does not involve a significant additional cost or workload to the IVF laboratory compared with the potential gain of balanced-diploid embryos obtainable. In fact, most 1PN embryos arrest their development even before the first cellular division and 2.1PN zygotes represent only a small portion of fertilized oocytes (0.7%; Supplemental Table 1). For the genetics laboratory, incorporation of the primers pool for the 40 highly variable SNPs in the qPCR-based CCT protocol or the parallel genotyping assessment by targeted NGS is not demanding and can be performed without any significant additional cost. Accordingly, the time and costs associated with the implementation of this combined aneuploidy and second-line ploidy analysis does not represent a major barrier for the systematic application of AFO assessment and clinical use.

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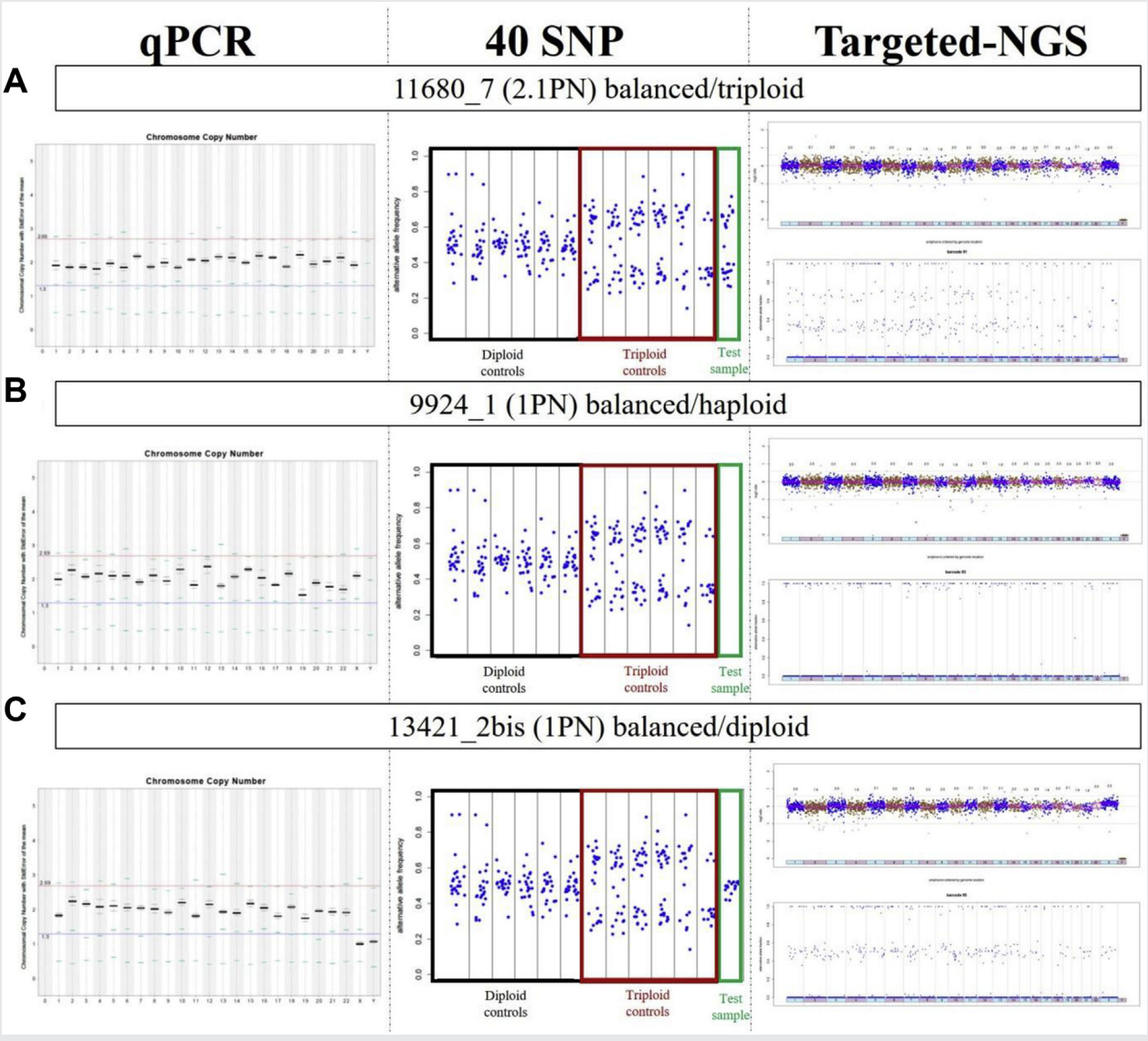
SUPPLEMENTAL FIGURE 1



Copy number analysis and ploidy determination by allelic ratios of informative single nucleotide polymorphisms of **(A)** a balanced-triploid, **(B)** a balanced-haploid, and **(C)** a balanced-diploid blastocyst. The first-line genetic scheme entails a chromosome copy number analysis conducted by means of quantitative polymerase chain reaction (qPCR) and a secondary ploidy assessment based on the 40–single-nucleotide polymorphisms (SNPs) method on the same trophectoderm (TE) biopsy. For some cases, a third approach based on targeted next-generation sequencing (NGS) on a second trophectoderm biopsy was also adopted for confirmation. For qPCR, the black bars represent the copy number values calculated for each specific chromosome by comparing the qPCR data with a cluster of data from normally fertilized unbalanced male blastocysts. The green bars represent the thresholds for nullisomy, monosomy, and trisomy from the bottom to the top, respectively for each specific chromosome. For the 40-SNP approach, each column identifies a sample (the first six columns, outlined in black, report the results from diploid control samples, and the second six columns, outlined in red, report the results from triploid control samples), and each blue spot represents the B-allele frequency for all the highly polymorphic informative SNPs assessed. The last column, outlined in green, shows the results obtained from the test sample. For targeted NGS, the profile plot on the top represents the chromosomal analysis, and the graph on the bottom represents the genotyping profile based on an average of 2,400 SNPs across the genome. In triploid samples, genotyping analysis reveals a 2:1 ratio [e.g., (A) for both the 40-SNP and the targeted-NGS approaches], and for haploid samples a loss of heterozygosity is observed [e.g., (B) for both the 40-SNP and the targeted-NGS approaches]. PN = pronuclei.

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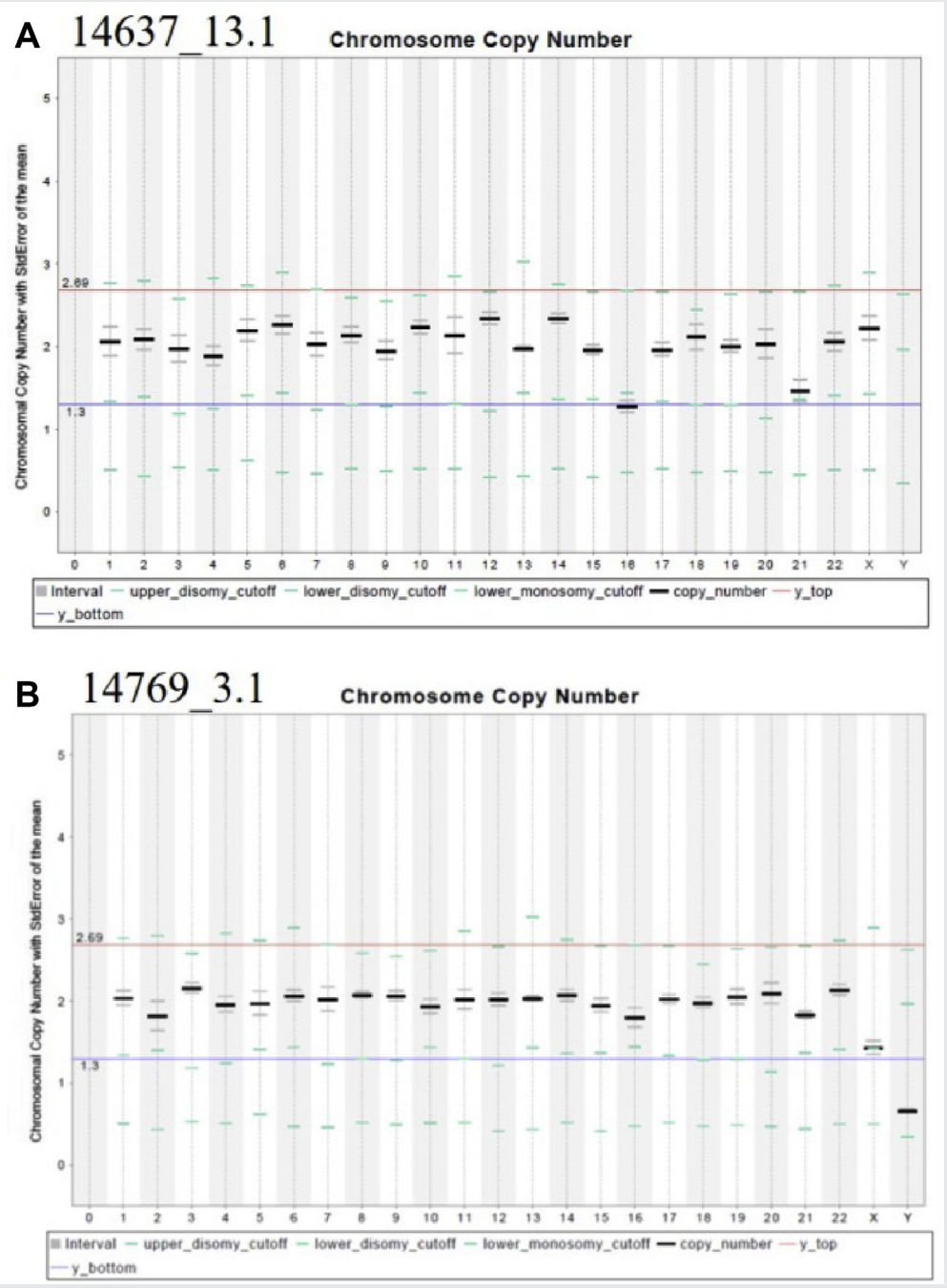
SUPPLEMENTAL FIGURE 2



Examples of quantitative polymerase chain reaction (qPCR) plots from **(A)** an unbalanced female triploid blastocyst and **(B)** a balanced male triploid embryo. The black bars represent the copy number values calculated for each specific chromosome by comparing the qPCR data with a cluster of data from 2PN balanced male diploid blastocysts. The green barsrepresent the thresholds for nullisomy, monosomy, and trisomy from the bottom to the top, respectively, for each specific chromosome.

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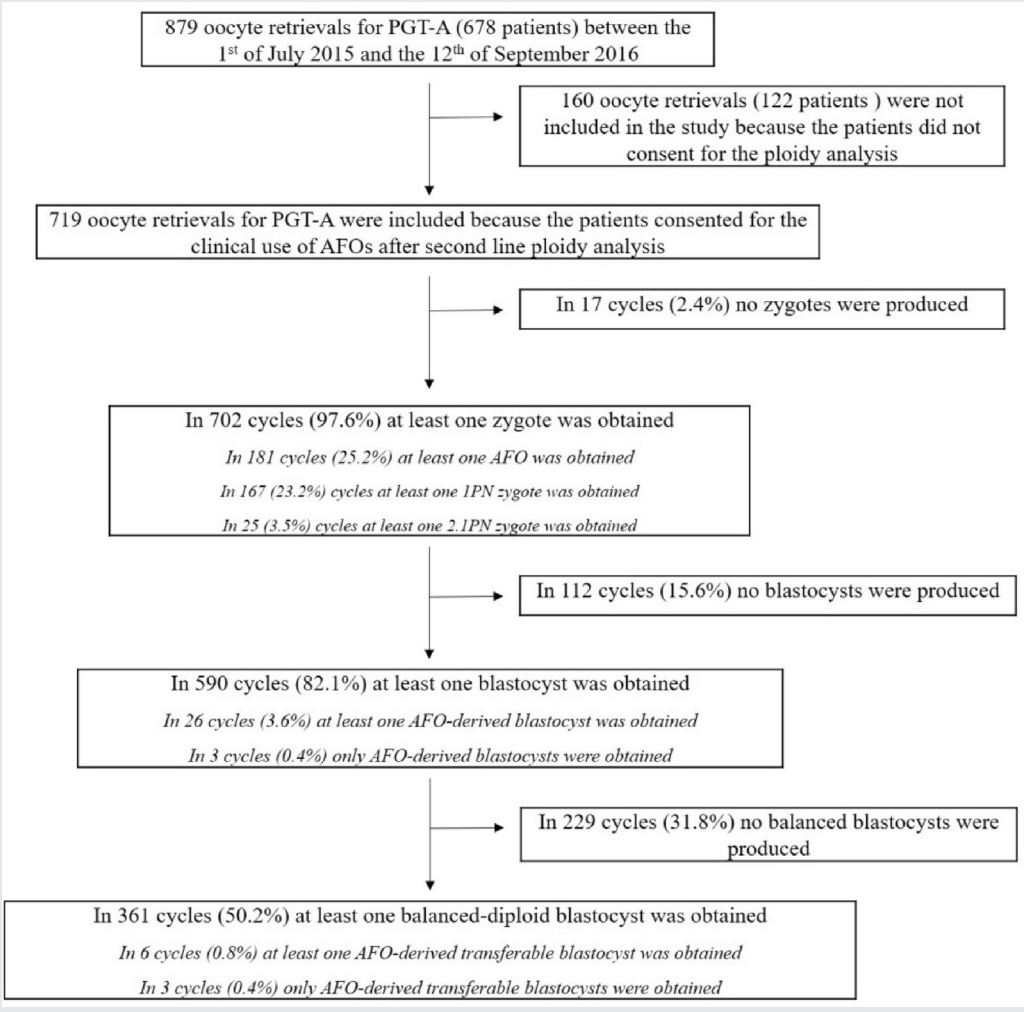
SUPPLEMENTAL FIGURE 3



Flow chart of the clinical implementation study. AFO, abnormally fertilized oocyte; PGT-A, preimplantation genetic testing for aneuploidies; PN, pronuclei.

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