Diminished effect of maternal age on implantation after preimplantation genetic diagnosis with array comparative genomic hybridization

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Objective: To assess the relationship between maternal age, chromosome abnormality, implantation, and pregnancy loss.
Design: Multicenter retrospective study.
Setting: IVF centers in the United States.
Patient(s): IVF patients undergoing chromosome screening.
Intervention(s): Embryo biopsy on day 3 or day 5/6 with preimplantation genetic diagnosis (PGD) by array comparative genomic hybridization.
Main Outcome Measure(s): Aneuploidy, implantation, pregnancy, and loss rates.
Result(s): Aneuploidy rates increased with maternal age from 53% to 93% for day 3 biopsies and from 32% to 85% for blastocyst biopsies. Implantation rates for euploid embryos for ages <35–42 years did not decrease after PGD: ranges 44%–32% for day 3 and 51%–40% for blastocyst. Ongoing pregnancy rates per transfer did not decrease for maternal ages <42 years after PGD with day 3 biopsy (48.5%–38.1%) or blastocyst biopsy (64.4%–54.5%). Patients >42 years old had implantation rates of 23.3% (day 3), 27.7% (day 5/6), and the pregnancy rate with day 3 biopsy was 9.3% and with day 5 biopsy 10.3%.
Conclusion(s): Selective transfer of euploid embryos showed that implantation and pregnancy rates were not significantly different between reproductively younger and older patients up to age 42 years. Some patients who start an IVF cycle planning to have chromosome screening do not have euploid embryos available for transfer, a situation that increases with advancing maternal age. Mounting data suggests that the dramatic decline in IVF treatment success rates with female age is primarily caused by aneuploidy. (Fertil Steril® 2013;100:1695–703. ©2013 by American Society for Reproductive Medicine.)
Key Words: Preimplantation genetic screening, embryo biopsy, aneuploidy, elective single-embryo transfer

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Aneuploidy in spontaneous abortions has been reported to be as high as 70% in some studies (1–7). Large studies on human embryos have shown much higher rates of chromosome abnormalities than seen in established clinical pregnancies, suggesting that an elimination of aneuploidies takes place (8–13). Indeed, a recent study shows a decrease in chromosome abnormalities from cleavage stage to

blastocyst stage (12), indicating that the loss of chromosomally abnormal embryos begins during preimplantation development. The high frequency of aneuploid preimplantation embryos may provide an explanation for the low implantation and birth rates achieved by assisted reproductive treatments (ART), especially for women of advanced reproductive age. For example, of 7,753 embryos analyzed by microarray comparative genome hybridization (aCGH), from 30% (maternal age <35 years) to 87% (maternal age >42 years) of blastocysts analyzed harbored numeric chromosome abnormalities, most of which are considered to be incompatible with establishment of a viable pregnancy (12). Of course, women in the younger bands of reproductive age (i.e., <35 years) have aneuploidy rates of ~40% (12) and would be expected to have fairly high implantation rates.

Following successful embryo biopsy and pregnancy for monogenic diseases (14–16), preimplantation genetic diagnosis (PGD) for aneuploidy, also known as preimplantation genetic screening (PGS), was first attempted by Munné et al. (17). By screening embryos for chromosome abnormalities and transferring only those found to be euploid, the expectation was that increased implantation and pregnancy rates and reduced pregnancy loss rates would be observed. The first techniques used for PGS were polar body biopsy or cleavage-stage blastomere biopsy followed by fluorescence in situ hybridization (FISH) analysis. This version of PGS can be called first-generation PGS. Initial studies with first-generation PGS suggested that implantation rates increased and loss rates decreased (18–28). However, other studies, including several randomized controlled trials (RCTs), showed no benefit (29–36) or, worse, a negative impact on implantation, pregnancy, or loss rates (37, 38).

The discrepancies between these studies have been debated extensively, and are probably due to technical differences (39). Specially, the Mastenbroek et al. (37) manuscript showed that in their hands, 60% of implantation potential was lost solely owing to the performance of cleavage-stage biopsy in their laboratory. Later studies by Treff et al. (40), presented at the American Society for Reproductive Medicine (ASRM), have shown that cleavage-stage biopsy has the potential to reduce implantation rates, a negative effect that has not been seen for blastocyst biopsy. Therefore, the selection of euploid embryos may or may not compensate for the damage caused by the biopsy. In addition, the use of FISH suffers from a number of issues inherent in the technique, including a limited number of unique fluorescent colors allowing only a handful of chromosomes to be tested in each case (5–12); worse, cell fixation and FISH interpretation are not standard techniques and are technician dependent. For example, reported FISH error rates have ranged from 50% (41) to 3.7% (42). First-generation PGS provided benefits in many published reports, but it was unable to convincingly prevent the deterioration in IVF outcomes, especially implantation rate, seen with advancing maternal age.

It became clear that a new version of PGS was needed. An ideal technique would allow for the simultaneous analysis of all 24 chromosomes (autosomes 1–22, X, and Y) and would be robust and less prone to technical issues that could lead to errors and misdiagnosis than earlier FISH methods. Additional benefit was recognized with the ability to reliably grow, biopsy, and cryopreserve embryos at the blastocyst stage. Biopsy at the blastocyst stage is thought to be less detrimental to embryo development.

The first comprehensive analysis technique appearing after FISH was comparative genomic hybridization (CGH) (43–45), but it was challenging to put into clinical practice because it needed at least three full days for the analysis to be completed. This meant that application to cleavage-stage embryos had to be combined with cryopreservation. However, the slow-freezing methods available at the time were detrimental to biopsied day 3 embryos (46). Blastocyst biopsy was perfected clinically by McArthur et al. (47). Finally, the advent of vitrification, which permitted the safe cryopreservation of biopsied embryos (48), allowed all the components for second-generation PGS to be assembled: complete chromosome screening (via CGH); less damaging embryo biopsy (at the blastocyst stage); and enough time to carry out the test (afforded by vitrification). The first clinical complete chromosome analysis of blastocysts was published by Schoolcraft et al. (49), achieving exceptional implantation rates in a patient group including advanced reproductive age and multiple IVF failures. CGH was later displaced by more automated techniques, such as aCGH (50–53), single-nucleotide polymorphism (SNP) arrays (54–56), and quantitative fluorescent polymerase chain reaction (qPCR) (57). Of these techniques, aCGH (58) and qPCR (59) have been shown in RCTs to improve pregnancy rates.

The present study aimed to determine if aCGH followed by selective transfer of euploid embryos has the potential to arrest the age-related decline in implantation rates observed in IVF cycles. If so, the underlying hypothesis of PGS would be finally proven, that is, that the bulk of implantation failure and pregnancy loss with advancing maternal age is due to chromosome abnormalities and can be prevented by replacing euploid embryos. Although it is possible that the retrospective nature of this article must allow for some bias in the data, the fact that the data is derived from a number of IVF centers around the United States should allow for some conclusions to be drawn.

MATERIALS AND METHODS

The data set included patients that underwent PGS by aCGH at a number of fertility clinics around the United States and for which it was possible to obtain follow-up information. All IVF clinics that sent patient samples to the reference laboratory were asked to follow up each case with information including the number of embryos transferred (along with the identification number of each embryo) as well as data on pregnancy test, presence of a fetal sac and/or heartbeat, information on any pregnancies lost following a positive test, and the outcome of prenatal diagnosis and/or live birth. Only clinics that supplied 100% data from all cycles referred for testing were included in the present study (n = 101). The database used for this study prevented directly or indirectly identifying individual patients. Therefore, the study was determined to be

exempt from approval by the Western Institutional Review Board in Olympia, Washington. According to the common rule 45 CFR 46.101(b)(4), exemptions include "research, involving the collection or study of existing data, documents, records, pathologic specimens, if these sources are publicly available or if the information is recorded by the investigator in such manner that subjects cannot be identified, directly or through identifiers linked to subjects."

Briefly, after initial infertility testing, each patient's ovaries were stimulated with the use of gonadotropins to produce multiple mature oocytes, which were retrieved with the help of ultrasound guidance. Oocytes were inseminated with the use of conventional IVF techniques or intracytoplasmic sperm injection (ICSI) (60). Centers were allowed to choose the most appropriate method of insemination based on patient criteria. Before clinical application of the chromosome screening test, experiments were undertaken in the laboratory to assess the ability of the whole-genome amplification method to amplify sperm. It was determined that sperm do not decondense and amplify at the temperatures and cycling parameters of the whole-genome amplification method used in this study (Reprogenetics, unpublished data). Approximately 18 hours after insemination, fertilization was assessed by microscopic evaluation. Normally fertilized embryos were further cultured until either day 3 or day 5/6 of embryo development, usually with sequential media; however, some laboratories used a single culture medium for all phases of embryo growth.

Embryo biopsy took place either on the morning of day 3 of embryo development or at blastocyst stage, which was usually on day 5 but on day 6 for some embryos growing at a slower rate. Biopsy was performed with laser-assisted hatching followed by removal of a single cell (cleavage-stage biopsy) or a clump of trophectoderm tissue (blastocyst biopsy). The biopsied material was washed in clean biopsy wash medium (Reprogenetics). After washing, the cell or tissue was placed in the supplied transport tube labeled with the patient initials and embryo number and kept on ice until being shipped to the genetics laboratory for testing. All samples were shipped by custom courier or commercial shipping companies and delivered to the genetics laboratory either the same day or early the next morning for testing. Individual IVF centers were allowed to determine the optimum moment of biopsy based on patient parameters and laboratory parameters.

Microarray-CGH was performed as previously described (51). Briefly, following sample receipt in the lab, each tube was opened in a dedicated DNA-amplification clean room, under laminar flow conditions, and the amplification reagents were added (Sureplex, Rubicon Genomics/Bluegnome; CPC4, Capital Park). After amplification according to the manufacturer's instructions, each sample was loaded onto an agarose gel to check for amplification. A smear of DNA, observed on the gel after electrophoresis, was indicative of positive amplification. All samples that were positive for DNA amplification (98% and 99.1% of day 3 and blastocyst biopsy samples, respectively) were taken to the fluorescent-labeling steps. Labeling was performed according to the manufacturer's recommendations with Cy3 dye for test DNA and Cy5 dye for reference male DNA (Bluegnome). After labeling, embryo biopsy samples and reference DNA samples (Bluegnome; used according to manufacturer's instructions) were separately denatured at 74°C before being mixed together and added to each microarray. For this study, version 2 of 24sure microarrays were used (Bluegnome). Microarrays were hybridized at 47°C for $\geq$4 hours or overnight in a humidified chamber. The length of hybridization time varied depending on the number of samples in the laboratory on any given day, the time that samples were received during the day, and the staffing levels during different times. During validation of the array in the laboratory, hybridization times as short as 4 hours and as long as 16 hours (overnight) were tested with no differences noted. On the basis of these results, hybridization for $\geq$4 hours and $\leq$16 hours is deemed to be interchangeable (Reprogenetics, unpublished data). After hybridization, each microarray was washed as follows: 10 minutes in 2× saline sodium citrate (SSC)/0.05% Tween-20 at room temperature, 10 minutes in 1× SSC at room temperature, 5 minutes in 0.1× SSC at 60°C and 2 minutes in 0.1× SSC at room temperature.

Each microarray was then scanned for green fluorescence at 632 nm and red fluorescence at 587 nm. Raw images were loaded automatically into Bluefuse software (Bluegnome), allowing for automated evaluation of fluorescent signals. Each sample was scored by a trained technologist who assessed all 24 chromosomes, noting all gains and losses as well as determining the sex of each sample. A second technologist then scored the sample blindly, with no knowledge of the initial score by the first technologist. A final score for each sample was assigned by comparing the scores of technologist 1 and technologist 2. Any discrepancies were noted and were adjudicated by a third technologist and/or the laboratory supervisor or director. Discrepancies were rarely noted between scorers (approximately one discrepancy per 500 embryos scored was noted). Once results for all samples from each patient were finalized, a diagnostic report was prepared and shared with the referring physician ahead of embryo transfer.

Transfer of euploid embryos typically took place on the morning of day 5 for cleavage-stage biopsy. For samples biopsied on day 5 or day 6 of embryo development, resulting blastocysts were cryopreserved with the use of either vitrification or slow cooling techniques. For blastocyst biopsy cases, euploid embryos were transferred in a controlled endometrial development cycle into a uterus free of gonadotropin stimulation. Clinical decisions were left with the individual IVF centers, including which embryos and how many embryos to transfer. Follow-up information regarding those clinical decisions was shared with the genetics laboratory through secure e-mail exchanges and/or telephone calls to individual IVF centers.

For statistical analysis, the existence of an association between age and outcome was tested with the use of contingency chi-square ($2 \times 2 \times 5$) analysis (χ^2). Expectations for each outcome group were determined by the observed incidence of the positive outcome (pooled for each day of biopsy regardless of age) multiplied by the number of patients within the biopsy day (3 or 5/6) and age group (<35, 35–37, 38–40, 41–42, or >42 years). Likewise, expectations of the negative

outcome for each biopsy day and age group were calculated with the use of (1 − incidence of the positive outcome) multiplied by the number of patients within the biopsy day (3 or 5/6) and age group (<35, 35–37, 38–40, 41–42, or >42 years).

For comparisons of day 3 versus day 5/6 biopsies, analyses were performed with the use of day 5/6 results as the expected for day 3 observations. When this is done, only the ten fields (2 × 5) for outcome × age are tested for one biopsy period. Expectations are determined by using the incidence of positive outcome (for day 5/6 biopsy in each age category) times the number of patients or embryos in the same age category for day 3 biopsy. Likewise, the incidence of negative outcome (for day 5/6 biopsy in each age category) times the number of patients or embryos in the same age category for day 3 biopsy results in the expected value for negative outcome. Degrees of freedom are not reduced as much as the contingency tests, because there is no reduction in degrees of freedom when expected frequencies are from outside the analyzed data.

The goal of this study was to determine if implantation rates across different maternal ages changed after PGS with embryo biopsy on day 3 (cleavage stage) or blastocyst biopsy followed by aCGH. Within each biopsy group, the data were further broken down into the Society for Assisted Reproductive Technology–recommended groups: <35, 35–37, 38–40, 41–42, and 43–44 years of age at the time of cycle start. Within each group, implantation rate as defined by detection of a fetal sac on ultrasound, ongoing pregnancy rate per transfer, and aneuploidy rate (as defined as an abnormal number of chromosomes in the sample) were assessed. In addition, pregnancy loss rate, as defined by loss of a clinical pregnancy following a positive pregnancy test, also was assessed.

RESULTS

A total of 451 cycles of cleavage-stage biopsy and 462 cycles of blastocyst biopsy were included in the study, with 3,412 and 2,467 embryos analyzed, respectively. No differences were noted in patient parameters between patients with day 3 and day 5/6 biopsy.

As can be seen in Table 1, the implantation rate for the youngest age group, <35-year-old patients, was 40.6% for day 3 biopsy and 51.1% for blastocyst biopsy. For the 35–37-year-old patient group, the implantation rate for biopsy on day 3 was 43.6% and for blastocyst biopsy 54.2%. For the 38–40-year-old patient groups, the implantation rate for biopsy on day 3 was 42.1% and for blastocyst biopsy 47.2%. And for the 41–42-year-old patients, the implantation rate for biopsy on day 3 was 31.6% and for blastocyst biopsy 40.4%. There were <40 replacements for patients ≥43 years old for patients with biopsy on day 3 (n = 20) or blastocyst stage (n = 16). For the ≥43-year-old patient group, the implantation rate for biopsy on day 3 was 23.3% and for blastocyst biopsy 27.7%. There was no significant association between implantation rates and maternal age for biopsy at either cleavage stage or blastocyst stage ($.1<P<.2$). The overall implantation rate was lower when embryos were biopsied at day 3 (39.6%) compared with embryos biopsied at the blastocyst stage (49.2%; $P<.005$). Of note, for the day 3 biopsy group, 137 cycles did not result in an embryo transfer, 111 owing to no euploid embryos and 26 for other reasons, whereas for trophectoderm biopsy, 119 cycles did not result in a transfer, 73 owing to no euploid embryos, and 46 for other reasons.

Although implantation/pregnancy rates are critical factors in infertility treatment, and are often the main statistics focused on by patients when choosing a clinic, another important measure of IVF success is maintenance of the pregnancy through to live birth. Toward this end, we also looked at pregnancy loss rates for each age group and time of biopsy to determine if there were any differences (Table 2). No significant difference was noted between loss rates for any age group or time of biopsy, averaging 9.9% for day 3 and 7.9% for blastocyst biopsy. The incidence of clinical pregnancy loss was significantly less after transfer of embryos selected

TABLE 1

Comparison of implantation rate and aneuploidy rate between biopsy at day 3 or day 5/6 by SART age group.

Day 3 biopsy			Day 5/6 biopsy		
Age group (y)	Implan.[a,b] (+Sac)	Aneuploidy rate[c,d]	Age group (y)	Implan.[a,b] (+Sac)	Aneuploidy rate[c,d]
<35	40.6% (73/180)	53.1% (530/999)	<35	51.1% (119/233)	31.7% (306/966)
35–37	43.6% (44/101)	68.2% (420/616)	35–37	54.2% (65/120)	44.2% (237/536)
38–40	42.1% (59/140)	73.7% (659/894)	38–40	47.2% (59/125)	43.1% (324/751)
41–42	31.6% (18/57)	85.8% (460/536)	41–42	40.4% (19/47)	76.3% (200/262)
>42	7/30	92.6% (340/367)	>42	5/18	84.8% (112/132)
P value	NS	<.001	*P* value	NS	<.001
Total	39.6% (201/508)	70.6% (2409/3412)	Total	49.2% (267/543)	47.8% (1179/2467)

Note: Implan. (+Sac) = Implantation rate as measured by the presence of a fetal sac by ultrasound; NS = not significant.

[a] The existence of an association between age and implantation rate was tested using Contingency Chi Squared (2 X 2 X 5) analysis (χ^2). χ^2 was 12.4 with 9 degrees of freedom ($.1<P<.2$). The lack of significance of this χ^2 value indicates that there was no significant association of implantation rate with age groups following embryo biopsy and preimplantation genetic screening when only euploid embryos were transferred.

[b] Associations between implantation and day 3 biopsy versus day 5/6 biopsy were tested using Chi Squared Analysis (2 X 5). χ^2 was 16.1 with 5 degrees of freedom ($.0005<P<.01$) when day 3 observations were tested using day 5/6 expectations. The significance of the χ^2 values indicates that the incidence of implantation was associated with biopsy day.

[c] The existence of an association between age and aneuploidy rate was tested using Contingency Chi Squared (2 X 2 X 5) analysis (χ^2). χ^2 was 559 with 9 degrees of freedom ($P<.001$). The significance of this χ^2 value indicates that there was a significant association between of the incidence of aneuploidy and patient age.

[d] Associations between ploidy and day 3 biopsy versus day 5/6 biopsy were tested using Chi Squared Analysis (2 X 5). χ^2 was 739 with 5 degrees of freedom ($P<.001$) when day 3 observations were tested using day 5/6 expectations. The significance of the χ^2 values indicates that the incidence of aneuploidy was associated with biopsy day.

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TABLE 2

Comparison of pregnancy loss rate between biopsy at day 3 or day 5/6 by SART age group.

Day 3 biopsy		Day 5/6 biopsy		IVF[a]
Age group (y)	Loss rate[b,c,d]	Age group (y)	Loss rate[b,c,d]	Loss rate[d]
<35	12.5 % (7/56)	<35	7.6 % (7/92)	13.0% (14193/109018)
35–37	11.4 % (4/35)	35–37	9.8 % (5/51)	17.9% (8554/47798)
38–40	8.3 % (4/48)	38–40	8.2 % (4/49)	26.0% (9099/34976)
41–42	0/16	41–42	1/19	38.1% (4165/10923)
>42	1/6	>42	0/4	52.7% (1624/3079)
Total	9.9 % (16/161)	38–40	7.9% (17/215)	18.3% (37635/205794)

[a] Data for the incidence of clinical pregnancy loss were assembled from the SART reports for years 2006–2011 for patients using their own oocytes in fresh IVF cycles. These numbers indicate the number of clinical pregnancies that did not result in live birth.
[b] The existence of an association between age and pregnancy Loss was tested using Contingency Chi Squared (2 X 2 X 5) analysis (χ^2). χ^2 was 3.5 with 9 degrees of freedom ($P>.25$). The lack of significance of this χ^2 value indicates that there was no significant association of pregnancy loss with patient age following transfer of only euploid embryos.
[c] The existence of an association between day of biopsy and pregnancy loss was tested using Contingency Chi Squared (2 X 2 X 4) analysis (χ^2). (Note patients in the 40–41 and >42 groups were pooled for this analysis due to the lack of pregnancy losses in both of the 41–42 year old groups and the associated expectation of 0). χ^2 was 2.4 with 4 degrees of freedom ($P>.25$). The lack of significance of this χ^2 value indicates that there was no significant association between incidence of pregnancy loss and biopsy day following transfer of only euploid embryos.
[d] The incidence of clinical pregnancy loss per age group for biopsy patients was compared with the incidence of loss among IVF patients using χ^2analysis, in which the incidence of pregnancy loss for IVF patients was used as the expectation for both biopsy days within each age group. Patients above the age of 40 were consolidated (41–42 plus >42), since there were small expectations in the >42 group. (χ^2=48.0 with 12 degrees of freedom, $P<.001$). The significance of this χ^2 indicates that the incidence of pregnancy loss following preimplantation genetic screening (either day 3 or day 5) was significantly less than the incidence of pregnancy loss in IVF patients reported to SART.

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for euploidy than is observed in a general IVF population ($P<.001$; Table 2). Of note, it was possible to analyze the chromosomes of only four of the pregnancy losses. One was 46XX, one was trisomy 16, one was tetraploid 96,XXYY, and one was trisomy 22. The trisomy 16 miscarriage was reanalyzed by aCGH with the use of DNA from the original biopsy and was found to be euploid. A number of causes of this finding could be postulated, including mosaicism in the embryo, lab error (IVF center or reference center) or some other error. The trisomy 22 was determined to be caused by human error, exacerbated by a software design problem, since then corrected. In the newest version of the array, each sample is compared with both a normal male and a normal female sample. This method allows for better discrimination between gains or losses of chromosome 22 and gains or losses of the X chromosome. Regarding the polyploid miscarriage, aCGH cannot detect this type of abnormality. This fact is clearly stated as a limitation of the test in the clinical report back to the referring physician.

Because implantation rates and miscarriage rates were not associated with maternal age, we would expect that pregnancy rates per transfer would not be associated either. Indeed, provided that there were euploid embryos for transfer, ongoing pregnancy rates per transfer were not associated with maternal age (Table 3). Ongoing pregnancy rate per transfer in the day 3 biopsy group ranged from 48.5% to 38.1% and in the blastocyst biopsy group from 64.4% to 54.5% for ages <35–42 years. Again, no statistically significant association of ongoing pregnancy per transfer with patient age was seen ($.05<P<.10$). For patients ≥43 years old, ongoing pregnancy rate for day 3 biopsy was 9.3% and for biopsy on day 5 was 10.3%.

As expected, aneuploidy rates increased as maternal age advanced (Table 1), no matter the day of biopsy. For day 3

TABLE 3

Comparison of ongoing pregnancy rate per embryo biopsy cycle and per transfer between day 3 biopsy or blastocyst biopsy.

Day 3 biopsy			Day 5/6 biopsy		
Age group (y)	OP/BX cycle[a,b]	OP/transfer[c,d]	Age group (y)	OP/BX cycle[a,b]	OP/transfer[c,d]
<35	43.4% (49/113)	48.5% (49/101)	<35	57.4% (85/148)	64.4% (85/132)
35–37	40.8% (31/76)	50.8% (31/61)	35–37	47.4% (46/97)	59.0% (46/78)
38–40	34.4% (44/128)	48.9% (44/90)	38–40	39.1% (45/115)	53.6% (45/84)
41–42	20.0% (16/80)	38.1% (16/42)	41–42	28.6% (18/63)	54.5% (18/33)
>42	9.3% (5/54)	5/20	>42	10.3% (4/39)	4/16

Note: OP = Ongoing pregnancy as determined by the presence of a fetal sac at ultrasound investigation.
[a] The existence of an association between age and ongoing pregnancy per embryo biopsy cycle was tested using Contingency Chi Squared (2 X 2 X 5) analysis (χ^2). χ^2 was 64.3 with 9 degrees of freedom ($P<.01$). The significance of this χ^2 value indicates that there was a significant association of ongoing pregnancy per cycle start with age.
[b] Associations between ongoing pregnancy per biopsy cycle and day 3 biopsy versus day 5/6 biopsy were tested using Chi Squared Analysis (2 X 5). χ^2 was 14.6 with 5 degrees of freedom ($.01<P<.02$) when day 3 observations were tested using day 5/6 expectations. The significance of the χ^2 values indicates that the incidence of pregnancy per start was associated with biopsy day.
[c] The existence of an association between age and ongoing pregnancy per transfer was tested using Contingency Chi Squared (2 X 2 X 5) analysis (χ^2). χ^2 was 15.9 with 9 degrees of freedom ($.05<P<.10$). The lack of significance of this χ^2 value indicates that there was no significant association between the incidence of ongoing pregnancy per transfer and age groups.
[d] Associations between incidence of pregnancy per transfer and day 3 biopsy versus day 5/6 biopsy were tested using Chi Squared Analysis (2 X 5). χ^2 was 18.2 with 5 degrees of freedom ($.0025<P<.005$) when day 3 observations were tested using day 5/6 expectations. The significance of the χ^2 values indicates that the incidence of ongoing pregnancy per transfer was associated with biopsy day.

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biopsy, aneuploidy rates for patients <35 years were 53.1%, increasing up to 92.6% for patients ≥43 years ($P<.001$). For biopsy at the blastocyst stage, aneuploidy rates ranged from 31.7% for the <35-year-old patients to 84.8% for patients aged ≥43 years. When comparing aneuploidy rates between day 3 and blastocyst biopsies for each patient age group, rates for each age group were lower in the blastocyst biopsy group than for the day 3 biopsy group ($P<.001$).

Although implantation rates and pregnancy rates per transfer did not decrease with advancing maternal age, the number of cycles with no transfers owing to lack of euploid embryos was increased. When cycles without a transfer are included, it resulted in a decreasing ongoing pregnancy rate per cycle started with advancing maternal age. As can be seen in Table 3, ongoing pregnancy rates per cycle for the day 3 biopsy group decreased from 43.4% in patients <35 years old to 20% in patients 41–42 years old ($P<.001$), and for blastocyst biopsy they ranged, respectively, from 57.4% to 28.6% ($P<.001$).

DISCUSSION

The main conclusion of this study is that implantation rates of euploid embryos are the same at any maternal age up to 42 years, no matter the day of biopsy. Essentially, second-generation PGS abrogates the maternal age effect on implantation rates observed in ART, except, perhaps, for the most advanced reproductive ages. The lack of an age-associated trend when transfers were restricted to the use of euploid embryos indicates that aneuploidy is the main cause of decreased implantation rates with advanced maternal age when PGS is not used.

Of course, not all patients undergoing IVF, no matter their maternal age, should expect to have large numbers of blastocysts available for biopsy, especially on day 5/6 of development. Some patients in the older age groups may not even achieve biopsy on day 3, owing to limited numbers or quality of embryo; therefore, the results presented here concern only patients with enough embryos of sufficient quality to reach biopsy. However, the data presented here should allow clinicians to effectively counsel their patients about the pros and cons of embryo biopsy and chromosome screening.

Overall, blastocyst biopsy produced better outcomes than day 3 biopsy across all ages when looking at implantation rate and ongoing pregnancy rate per transfer. This may be due to less damage to the embryo during trophectoderm biopsy (40) or to the fact that blastocysts are better embryos because they have made the transition from maternal proteins to embryonic proteins and have undergone cell differentiation to achieve the blastocyst stage. These differences were less stark than expected from earlier studies (37, 40), perhaps owing to the fact that not all centers use identical biopsy techniques (day 3 or blastocyst) nor do they have a homogeneous patient population or apply PGS to the same groups of patients. The lack of standardization of biopsy methodology is a limitation of the present study. However, because data were obtained from multiple independent IVF clinics, it is likely that the results are, in general, representative of what can be expected when these methods are clinically applied. Another potential source of bias in this data is embryo quality. No measures of embryo quality at day 3 or day 5/6 were collected or measured in this study. Again, the large number of embryos from a number of clinics around the United States should allow for a general representation of embryo quality before and after biopsy.

The majority of cycles using blastocyst biopsy in this study involved cryopreservation and transfer in an unstimulated cycle. Though cryopreservation may entail some risk to the embryo, it also has the potential advantage that uterine receptivity may be enhanced in comparison with stimulated cycles. This may go some way to explaining the superior results obtained for embryos biopsied at the blastocyst stage. However, recent studies using blastocyst biopsy and PGS in conjunction with fresh embryo transfer have shown significant improvement in ongoing pregnancy rates (58, 59); therefore, enhanced receptivity might not be a key advantage of the blastocyst biopsy approach. On the other hand, not all laboratories attempting fresh transfer have had great success, and it seems that the timing of fresh day 6 transfer is critical (personal communication with a number of IVF centers in the United States, some using our reference laboratory and others using other methods). This may be further proof that the transfer window in stimulated cycles is not easy to gauge, because it may be advanced or delayed and, therefore, easily missed. As with first generation PGS using cleavage-stage biopsy, a lack of standardization regarding blastocyst biopsy and transfer could lead some clinics to obtain suboptimal results and conclude that the new PGS techniques are ineffective. Consequently, it is important that second-generation PGS is standardized quickly and best practice guidelines established.

In addition to achieving pregnancy following IVF treatment, maintaining pregnancy to delivery is vitally important. Loss rates in patients >35 years old are reported to be in the 20%–25% range (61), meaning that in this group one-fourth to one-fifth of all recognized pregnancies are lost before delivery. In the general IVF population reported in the United States, the incidence of pregnancy loss increased with age from 13.0% in patients <35 years old to as much as 38.1%–52.7% in patients >40 years old (Table 2). The pregnancy loss rate in the present study was 7.7% overall and was not associated with the age of the patient. Clearly, the transfer of euploid blastocysts after aCGH is leading to a decreased incidence of pregnancy loss in this population, which would be expected to lead to higher live birth rates after PGS testing and transfer of euploid embryos compared with blind transfer of embryos without screening.

Clinicians who offer chromosome testing have to make their patients aware that embryo transfer will not happen in every cycle, even in younger patients. Although the improved embryo selection afforded by methods such as aCGH can increase implantation rates across all age groups, it cannot improve pregnancy rates in patients that fail to produce at least one chromosomally normal embryo. Unfortunately, such patients become increasingly common with advancing maternal age, a problem that currently has no solution other than oocyte donation. As shown by Ata et al. (12), depending on cohort size and number of blastocysts available, 61% of women aged 40–42 years with four or fewer blastocysts will

have at least one euploid embryo available for replacement. The number of available euploid embryos increases with both cohort size and decreasing maternal age. However, as seen in the Yang et al. study (58), in good-prognosis patients ≤35 years old, 100% of patients in the testing arm (aCGH at the blastocyst stage) achieved transfer of one euploid embryo.

In our opinion, the use of second-generation PGS to achieve improved embryo selection and high implantation rates and the ability to reliably vitrify blastocysts mean that there is now little reason to replace more than one euploid blastocyst. In that respect, it is important to point out the excellent study of Yang et al. (58), a recent RCT using aCGH, which simultaneously demonstrated improved ongoing pregnancy rates and an absence of any multiple pregnancies.

In the present study, we were able to show that chromosome screening, here termed second-generation PGS, was able to mitigate the maternal age effect in all but the oldest category of IVF patients. Implantation rates and pregnancy rates remained static across the increasing maternal age groups, no matter whether biopsy was performed on day 3 or day 5/6 of embryo development in cycles that ended in a transfer. In addition, pregnancy loss rates remained low no matter the maternal age. The selective transfer of euploid embryos after chromosome screening allows for mitigation of the maternal age effect seen in IVF following the use of unselected embryos for patients that have at least one euploid embryo for transfer. Of course, as maternal age increases, the number of cycles without a transfer will also increase. Chromosome screening during IVF improves outcomes for most age groups by increasing implantation rates and decreasing pregnancy loss rates.

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