ORIGINAL ARTICLE: ASSISTED REPRODUCTION

Using outcome data from one thousand mosaic embryo transfers to formulate an embryo ranking system for clinical use

Manuel Viotti, Ph.D.,[a,b] Andrea R. Victor, M.S.,[a] Frank L. Barnes, Ph.D.,[a,b] Christo G. Zouves, M.D.,[a,b] Andria G. Besser, M.S.,[c] James A. Grifo, M.D., Ph.D.,[c] En-Hui Cheng, Ph.D.,[d] Maw-Sheng Lee, M.D., Ph.D.,[d,e] Jose A. Horcajadas, Ph.D.,[f] Laura Corti, M.Sc.,[g] Francesco Fiorentino, Ph.D.,[h] Francesca Spinella, Ph.D.,[h] Maria Giulia Minasi, M.Sc.,[i,j] Ermanno Greco, M.D.,[i,j] and Santiago Munné, Ph.D.[k]

[a] Zouves Foundation for Reproductive Medicine, Foster City, California; [b] Zouves Fertility Center, Foster City, California; [c] New York University Langone Fertility Center, New York, New York; [d] Lee Women's Hospital, Taichung, Taiwan; [e] Chung Shan Medical University, Institute of Medicine, Taichung, Taiwan; [f] Overture Life, New York, New York; [g] IRCCS San Raffaele Scientific Institute, Milan, Italy; [h] Eurofins Genoma Group, Molecular Genetics Laboratories, Rome, Italy; [i] European Hospital, Centre For Reproductive Medicine, Rome, Italy; [j] Villa Mafalda, Center For Reproductive Medicine, Rome, Italy; [k] Cooper Genomics, Livingston, New Jersey

Objective: To study how the attributes of mosaicism identified during preimplantation genetic testing for aneuploidy relate to clinical outcomes, in order to formulate a ranking system of mosaic embryos for intrauterine transfer.
Design: Compiled analysis.
Setting: Multi-center.
Patient(s): A total of 5,561 euploid blastocysts and 1,000 mosaic blastocysts used in clinical transfers in patients undergoing fertility treatment.
Intervention(s): None.
Main Outcome Measure(s): Implantation (gestational sac), ongoing pregnancy, birth, and spontaneous abortion (miscarriage before 20 weeks of gestation).
Result(s): The euploid group had significantly more favorable rates of implantation and ongoing pregnancy/birth (OP/B) compared with the combined mosaic group or the mosaic group affecting only whole chromosomes (implantation: 57.2% vs. 46.5% vs. 41.8%; OP/B: 52.3% vs. 37.0% vs. 31.3%), as well as lower likelihood of spontaneous abortion (8.6% vs. 20.4% vs. 25%). Whole-chromosome mosaic embryos with level (percent aneuploid cells) $<50\%$ had significantly more favorable outcomes than the $\geq 50\%$ group (implantation: 44.5% vs. 30.4%; OP/B: 36.1% vs. 19.3%). Mosaic type (nature of the aneuploidy implicated in mosaicism) affected outcomes, with a significant correlation between number of affected chromosomes and unfavorable outcomes. This ranged from mosaicism involving segmental abnormalities to complex aneuploidies affecting three or more chromosomes (implantation: 51.6% vs. 30.4%; OP/B: 43.1% vs. 20.8%). Combining mosaic level, type, and embryo morphology revealed the order of subcategories regarding likelihood of positive outcome.
Conclusion(s): This compiled analysis revealed traits of mosaicism identified with preimplantation genetic testing for aneuploidy that affected outcomes in a statistically significant manner, enabling the formulation of an evidence-based prioritization scheme for mosaic embryos in the clinic. (Fertil Steril® 2020;■:■–■. ©2020 by American Society for Reproductive Medicine.)
Key Words: IVF, preimplantation genetic testing, Next-Generation Sequencing, embryo, mosaicism

Discuss: You can discuss this article with its authors and other readers at https://www.fertstertdialog.com/posts/30988

Received July 11, 2020; revised November 17, 2020; accepted November 30, 2020.
S.M. is company officer and direct stockholder at Overture Life, and paid consultant at Cooper Surgical. J.A.G. is direct stockholder at Inception Fertility. M.V. has nothing to disclose. A.R.V. has nothing to disclose. F.L.B. has nothing to disclose. C.G.Z. has nothing to disclose. A.G.B. has nothing to disclose. E.H.C. has nothing to disclose. M.S.L. has nothing to disclose. J.A.H. has nothing to disclose. L.C. has nothing to disclose. F.F. has nothing to disclose. F.S. has nothing to disclose. M.G.M. has nothing to disclose. E.G. has nothing to disclose.
E.G. and S.M. should be considered similar in author order.
Reprint requests: Manuel Viotti, Ph.D., Zouves Foundation for Reproductive Medicine, 1241 East Hillsdale Blvd, Suite 100, Foster City, CA 94404 (E-mail: mviotti@zouvesfoundation.org).

Fertility and Sterility® Vol. ■, No. ■, ■ 2021 0015-0282/$36.00

https://doi.org/10.1016/j.fertnstert.2020.11.041

The assemblage of cells composing a human preimplantation embryo can contain different karyotype conformations. First, when all cells house the typical set of 46 chromosomes, an embryo is deemed "euploid". Second, an embryo is regarded as "aneuploid" when all its cells contain a particular chromosomal abnormality, such as segmental or whole-chromosome aneuploidy. As a third possibility, an embryo is deemed "mosaic" when two or more cell populations with different chromosomal content are present simultaneously. This phenomenon originates from post-zygotic errors of mitosis, such as nondisjunction or anaphase lagging, where sister chromatids fail to segregate correctly among two daughter cells (1). For purposes of preimplantation genetic testing for aneuploidy (PGT-A), mosaic embryos that contain a mix of euploid and aneuploid cells (hereafter simply referred to as "mosaic") have become highly relevant. As first described by Greco et al. (2) and later by others (3–7), embryos with a PGT-A result suggesting mosaicism can result in seemingly healthy pregnancies and births, albeit with lower success rates than euploid embryos.

Contemporary PGT-A operates on the premise that a cellular biopsy of the trophectoderm (TE) is representative of the entire blastocyst. Of the various existing molecular platforms, whole-genome amplification coupled with Next-Generation Sequencing (NGS) has a comparatively high dynamic range and resolution, and is considered the most appropriate system for detecting intrabiopsy mosaicism in the 20%–80% range between uniform euploidy and aneuploidy (4). Those thresholds coincide with the fact that a typical TE biopsy can contain five cells, and therefore anywhere from 1/5 to 4/5 abnormal cells in instances of mosaicism. Numerous reports have shown accurate detection of mosaicism in cell- and DNA-mixing experiments using whole-genome amplification–based NGS (4, 6–9). Consequently, a PGT-A grading system that considers mosaic results as a separate category has been proposed (10) and endorsed by professional societies such as Preimplantation Genetic Diagnosis International Society (11) and Controversies in Preconception, Preimplantation and Prenatal Genetic Diagnosis (12).

Nonetheless, concerns regarding the diagnosis and management of mosaicism in preimplantation embryos have been expressed. Suboptimal blastocyst biopsies may create false mosaic results (12), and technical background noise due to artifacts of amplification or sequencing could be indistinguishable from results consistent with mosaicism (13). Furthermore, there is subjectivity in diagnosing mosaicism with PGT-A because most contemporary analysis software is designed to classify samples as uniformly euploid or aneuploid, leaving the identification of mosaicism to the user. Other causes of artifactual mosaicism have been proposed, such as the cell cycle phase influencing readings resembling mosaic segmental abnormalities (14), although this effect appears to be minimized with contemporary, blastocyst-stage PGT-A methods (15).

Due to these concerns, still limited data on pregnancy outcome, and unknown potential risks associated with mosaic embryo transfer (16), some argue that mosaicism should not yet be reported (17) or that embryos classified as mosaic should not be transferred (18). In addition, doubts remain as to which characteristics of mosaicism correlate with clinical outcomes, such as proportion of aneuploid cells (mosaic level), nature of aneuploidy involved in the mosaicism (mosaic type), and identity of aneuploid chromosomes present. These points only can be addressed with larger datasets than currently published.

Here we present the analysis of 1,000 embryo transfers of mosaic blastocysts, showing that the proportion of abnormal cells and type of mosaic aneuploidy significantly affect clinical outcome. Identification of these mosaic characteristics with PGT-A in a clinical TE biopsy specimen, therefore, is appropriate and valuable for embryo selection. These data provide much needed evidence-based guidelines for ranking mosaic embryos in the clinic.

MATERIALS AND METHODS

Participating Centers and Data Collection

The following in vitro fertilization (IVF) clinics and PGT-A laboratories contributed data to this study: Zouves Fertility Center, Foster City, California, USA; New York University Langone Fertility Center, New York, New York, USA; Lee Women's Hospital, Taichung, Taiwan; IRCCS San Raffaele Scientific Institute, Milan, Italy; Eurofins Genoma Group, Molecular Genetics Laboratories, Rome, Italy; European Hospital, Centre For Reproductive Medicine, Rome, Italy; and Cooper Genomics, Livingston, New Jersey, USA.

"Mosaic embryos" are defined here as those where the PGT-A analysis of a TE biopsy specimen showed a profile consistent with mosaicism for one or more genomic regions. Participating centers contributed two data sets for a combined total of 1,000 mosaic embryos used in clinical transfers (Supplemental Table 1, available online). One set was from previously published reports (4–7), accounting for 425 mosaic embryos (comprising 42.5% of embryos in this study), each with additional unpublished information necessary for comprehensive analyses specific to this study. In one instance, samples from a previously published report were reprocessed with a different platform (NGS) for the purpose of the current study (6). The other set was new unpublished data, accounting for 575 mosaic embryos (comprising 57.5% of embryos in this study). Of the combined 1,000 mosaic embryos, 860 were used in single embryo transfers, 88 were used in double embryo transfers (DETs) together with another mosaic embryo, 50 were used in DETs together with a euploid embryo, two were used in DETs together with an untested embryo, and two mosaic embryos were used in a triple embryo transfer together with one mosaic and one euploid. In 94.6% of cases, a mosaic embryo was selected for transfer to a patient when no euploid embryo was available. In the remaining cases, a mosaic embryo was transferred together with a euploid (5.2%) or with an untested embryo (0.2%). For DETs and triple embryo transfers in which the embryos in a transfer resulted in different clinical outcomes, their identity could be deduced from the sex (through prenatal testing and/or at birth), otherwise they were excluded from the analysis.

For the control group, participating centers submitted clinical outcome data on embryos categorized as euploid (n = 5,561) for the same time period that mosaic embryo transfer data was collected. A weighted average implantation rate and ongoing pregnancy or birth rate was calculated for the control group, corrected for the proportion of mosaic embryos contributed by each center.

All patients in this study receiving an embryo transfer with known mosaic PGT-A results were advised previously by certified genetic counselors on the concept of embryonic chromosomal mosaicism and its potential clinical risks and consequences. A recommendation for prenatal testing was made in each case. The participating clinics were located in countries in which laws regulating IVF treatments reference reproductive rights and emphasize patient freedom of choice. Therefore, clinics could advise but not dictate decisions regarding embryo selection or prenatal and postnatal testing. Due to data anonymization for this study, patients were not contacted retroactively in instances where an embryo was reclassified from "euploid" to "mosaic" at reanalysis of the results (constituting 16.4% of embryos in the study) to communicate the change in embryo status. In the host countries of participating centers, the task of considering the ethical implications of a research project is performed by an Institutional Review Board (IRB). The analyses presented here were approved by the IRB of the Zouves Foundation (OHRP IRB00011505, Protocol #0002).

PGT-A

All embryos in this study underwent blastocyst-stage PGT-A using the same NGS-based platform VeriSeq (Vitrolife) and subsequent frozen embryo transfer. Trophectoderm biopsy specimens were collected using standard protocols and frozen until processing. Cell samples were lysed, and genomic DNA was fragmented randomly and amplified using the SurePlex DNA Amplification System (Vitrolife) according to the manufacturer's protocol. The whole-genomic amplified DNA product of each sample was processed to prepare a genomic DNA library using VeriSeq PGS workflow (Vitrolife). Purified DNA libraries were normalized to equalize the quantity of each sample in the final pool using VeriSeq's library normalization protocol. Equal volumes of normalized samples were pooled, denatured, and sequenced. The MiSeq Reagent Kit v.3 (Illumina) was used on a MiSeq System (Illumina). The sequencing data were analyzed using BlueFuse Multi Software (Vitrolife).

The participating centers in this study interpreted the resulting data uniformly: NGS profiles were defined as mosaic when displaying copy number counts in the 20%–80% range between chromosome monosomy and disomy or disomy and trisomy for any genomic region, as has been described previously (4, 12). Profiles <20% were considered euploid, and those >80% were considered aneuploid. All participating centers performed in-house validation experiments using DNA and/or cell mixes to produce accurately intermediate copy number profiles consistent with mosaicism, as published previously (4, 6, 7, 19). VeriSeq is validated to identify segmental gains and losses of 20 Mb or larger, but is capable of detecting segments as small as 1.8 Mb (20) in some genomic regions.

Definitions of Mosaic Traits, Clinical Indications, and Outcomes

Mosaic "level" referred to the inferred percentage of aneuploid cells in a TE biopsy specimen. For embryos with two or more mosaic chromosomal regions, the highest mosaic level value was considered for analysis.

Mosaic "type" referred to the nature of the chromosomal abnormality in the aneuploid cell compartment. Mosaic embryos with exclusively segmental abnormalities were called "single," "double," or "complex segmental," depending on the number of affected segments. Instances of mosaicism involving a single whole-chromosome aneuploidy (monosomy or trisomy) were considered "one chromosome" mosaics. Embryos with mosaicism in two whole chromosomes, or one whole chromosome and one segmental region, were considered "two chromosomes" mosaic. When mosaicism was present in more than two chromosomes, the mosaicism type was considered "complex," including combinations of whole chromosomes and segmental regions.

The clinical indications for PGT-A were defined as follows: advanced maternal age for maternal age >37 years at time of oocyte retrieval, repeat implantation failure for cases with three or more prior failed implantation upon transfer, recurrent spontaneous abortion/miscarriage for loss of two or more clinical pregnancies prior to week 20 of gestation, "PGT-M/-SR" for cases with familial genetic conditions undergoing concurrent PGT-A, "Other" (including male factor infertility and unexplained infertility), and "Good Prognosis" for cases of elective PGT-A with no specific clinical indication.

"Implantation" was defined by the presence of a gestational sac by endovaginal ultrasound at 3–5 weeks after transfer. When applicable, fetal heartbeat was monitored using endovaginal ultrasound at 6–8 weeks after transfer. Successful pregnancies were divided into "Ongoing Pregnancy" if there was active pregnancy or "Birth" if a baby was born. Any embryo that was positive for Implantation but negative for Ongoing Pregnancy/Birth (OP/B) before week 20 of gestation was considered to have succumbed to spontaneous abortion.

Statistics and Data Preparation

Statistical analysis was performed in Prism (GraphPad) and graphs were assembled in Illustrator (Adobe). Comparisons between groups with categorical outcome variables were performed according to sample size with a two-tailed chi-square test with Yates correction or a two-tailed Fisher exact test. Comparisons with quantitative outcome variables were performed with an unpaired, two-tailed *t* test. Trends were analyzed using linear regression or, in the case of ordinal variables, with a chi-square test for trend (Cochran-Armitage). For all analyses: $^{*}P<.05$; $^{**}P<.01$; $^{***}P<.001$; $^{****}P<.0001$; not significant, $P\geq.05$.

For normalization of the euploid group to the mosaic group by morphology (Supplemental Fig. 1, available online), we considered every combination possible in the Gardner and Schoolcraft system (21) for stage, inner cell mass (ICM) grade, and TE grade, and counted the number of mosaic embryos in the analysis group for every permutation. Next, we matched each count with an equal number of embryos from the euploid group, and each permutation with averaged clinical outcome rates of the euploid group (Supplemental Table 2, available online). This led to the calculation of rates of implantation and OP/B for a putative euploid group with identical morphological characteristics as the mosaic group.

For the generation of values in the Ranking Matrix, clinical outcome rates (Supplemental Table 3, available online) were normalized to the highest value in the euploid group. Only subgroups with $n \geq 10$ samples were included in the table, and all subgroups with $n < 10$ samples were indicated with "na". Stage 2 is not shown because none of the subgroups had the minimum 10 embryos for that stage.

RESULTS

Mosaic Embryos Experience Less Favorable Clinical Outcomes Than Euploid Embryos

Centers contributing data to this study had incidences of mosaic embryos ranging from 11.0%–25.7% in their general tested embryo population, with an average of 18.6% (Fig. 2A). Clinical outcome data were assembled from 5,561 euploid embryos and 1,000 mosaic embryos (Supplemental Table 1, available online), transferred between January 2015 and April 2020 at participating clinics. For mosaic embryos, in 83.6% of cases there was knowledge of the mosaic status prior to clinical transfer, whereas in 16.4% of cases the embryo was transferred under supposition of euploidy but post-transfer re-evaluation of the sequencing profile led to the embryo being assigned to the mosaic category (Fig. 2B). The following parameters showed similar overall patterns between the euploid and mosaic groups: day of biopsy, maternal age, and indication category for PGT-A (Fig. 2B). Compared with the euploid group, transferred mosaic embryos tended to originate from cycles producing fewer euploid blastocysts (on average 2.2 vs. 0.8) but more mosaic blastocysts (0.9 vs. 1.7) (Fig. 2B). Morphological evaluation with the Gardner and Schoolcraft system (21) indicated that, compared with the euploid group, the mosaic group had fewer grade A embryos (23.2% vs. 15.1%) and more ICM grade C embryos (14.4% vs. 19.4%), as well as more TE grade C embryos (18.9% vs. 28.9%) group (Fig. 2B).

Transfer of embryos in the euploid group resulted in an implantation rate of 57.2% and an OP/B rate of 52.3% (Fig. 2C). In comparison, the combined mosaic group had significantly lower rates of implantation (46.5%) and OP/B (37.0%) (Fig. 2C). When only considering whole-chromosome mosaic embryos (no segmental mosaics), outcome rates further decrerased for implantation (41.8%) as well as OP/B (31.3%) (Fig. 2C). Euploid embryos that implanted had an 8.6% likelihood of spontaneously aborting, whereas the likelihood was significantly higher for the combined mosaic group (20.4%) as well as the whole-chromosome mosaic group (25.0%) (Fig. 2C). Importantly, ~72% of the documented spontaneous abortions in mosaic embryos occurred early in the pregnancy, between observation of gestational sac (3–5 weeks after transfer) and fetal heart beat monitoring (6–8 weeks after transfer).

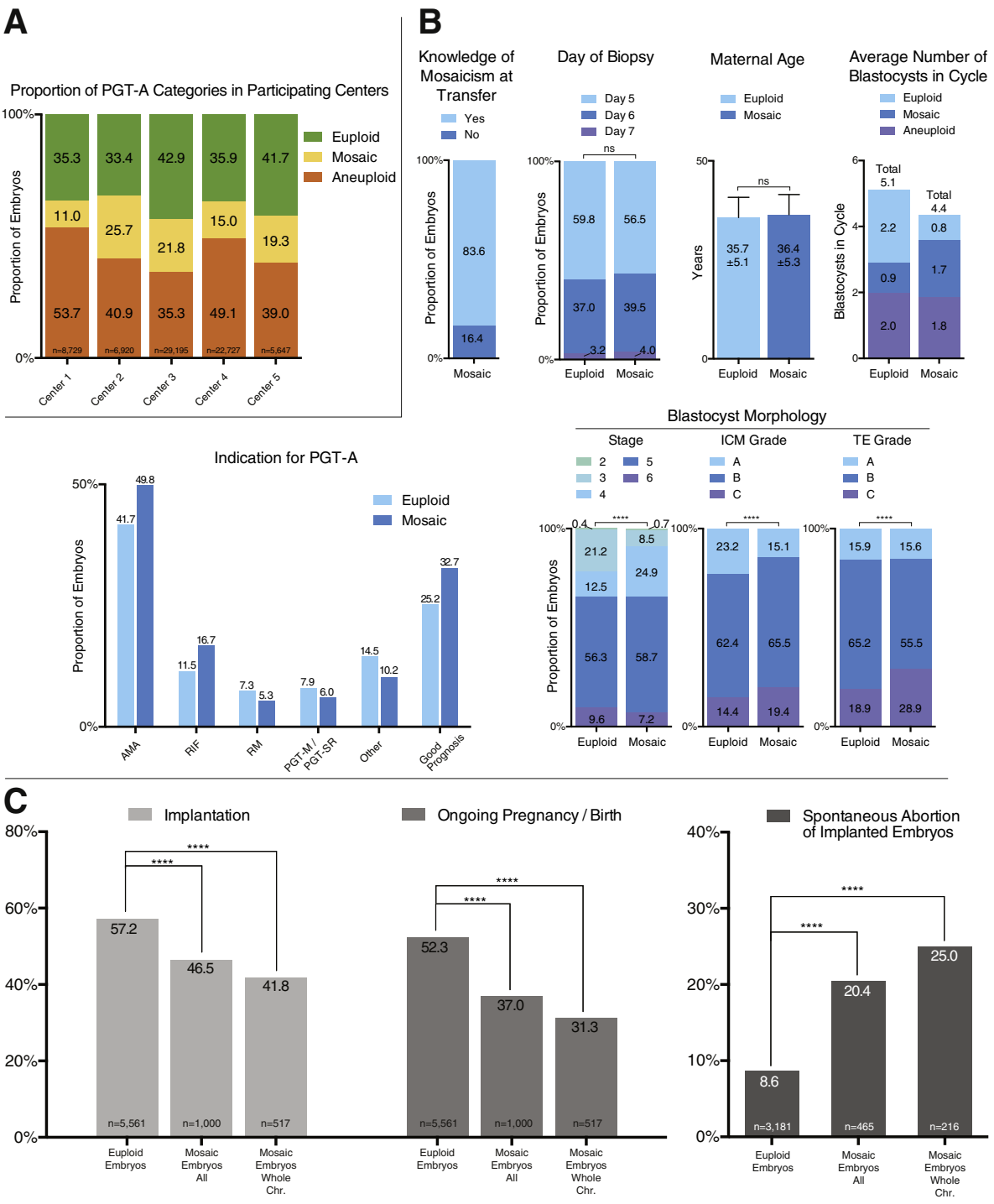
Since some mosaic embryos were transferred as DETs, we re-analyzed the data for mosaic embryos transferred exclusively as single embryo transfers ($n = 860$), observing that they resulted in significantly inferior clinical outcomes compared with the euploids (47.7% implantation, 37.8% OP/B) (Supplemental Fig. 1A, available online). Because euploid embryos typically are given first priority for transfer among embryos generated in a cycle, there was a possibility that mosaic embryo transfers usually occurred in patients who had undergone one or more prior failed transfers of euploid embryos from within the same cycle, meaning that different rates of clinical outcomes could be caused by a maternal-specific effect. To investigate that possibility, we analyzed outcomes for mosaic embryo transfers from cycles with no euploid embryos, in which mosaic embryos were the first to be transferred from within a cohort. That "no-euploid" mosaic group ($n = 517$) experienced significantly lower rates of implantation (44.1%) and OP/B (35.4%) compared with the euploid group (Supplemental Fig. 1A, available online).

Having noted that transferred mosaic embryos were, on average, of inferior morphological grade compared with the euploid group (Fig 2A), we proceeded to determine to what extent that difference was responsible for the decreased rates of clinical outcome observed in the mosaic group. To that end, we normalized the euploid group to the mosaic group by morphology (Materials and Methods). This lowered the rates of implantation and OP/B for euploid embryo transfers, and increased their rates of spontaneous abortion. However, comparing the morphology-normalized euploid group to the mosaic group revealed statistically significant differences in outcome, which were less favorable for the mosaic group (Supplemental Fig. 1B, available online). This suggested that differences in morphology could not account entirely for the inferior outcomes of mosaic embryos.

High-Level Mosaics Have Poorer Outcomes Than Low-Level Mosaics

We stratified the 1,000 mosaic embryos and analyzed how the level of mosaicism (i.e., the estimated percentage of abnormal cells that are mixed with normal cells) affected clinical outcomes. Data were plotted in 10% increments of mosaic level, representing a progressive increase in the proportion of aneuploid cells in the mix, and linear regression showed a statistically significant decrease in rates of implantation and OP/B for whole-chromosome mosaics, but not for segmental mosaics (Fig. 3A). We considered four different cutoffs to group embryos into "low" and "high" mosaicism. For whole-chromosome mosaics, neither 30% nor 40% as cutoffs yielded significant differences between the "low" and "high" groups (Supplemental Fig. 2, available online). However, using 50% or 60% as a cutoff resulted in significant differences, with 50% displaying the largest dissimilarities in clinical outcomes

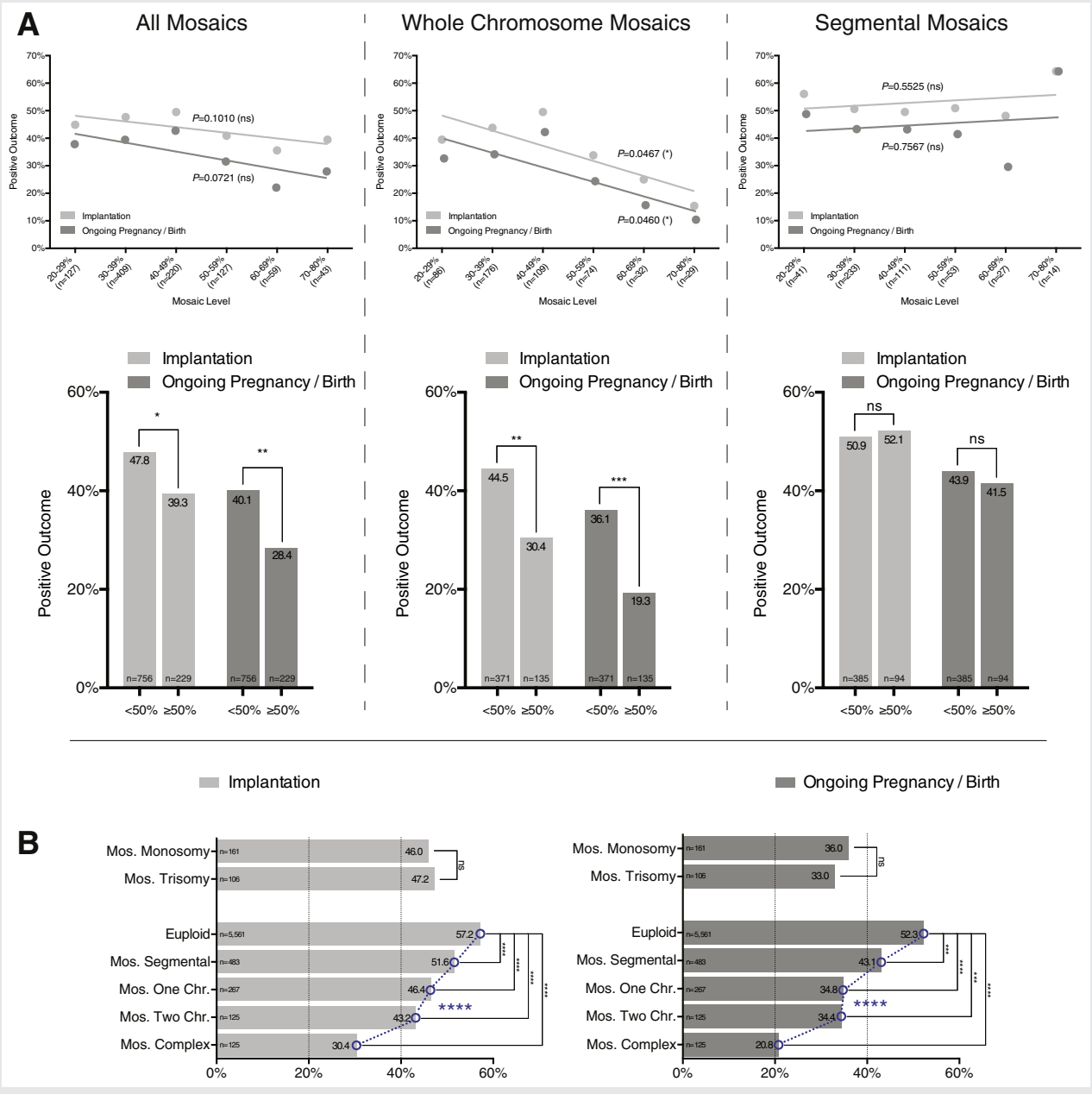
FIGURE 1



Characteristics and clinical outcomes of transferred euploid and mosaic embryos. (**A**) Proportion of embryos by preimplantation genetic testing for aneuploidy (PGT-A) category in participating centers. (**B**) Overview of data pertaining to 5,561 euploid embryos and 1,000 mosaic embryos included in this study. The "Maternal Age" graph depicts the mean with standard deviation (SD). In the "Indication for PGT-A" graph, the summed percentages exceed 100% because some embryos were in 2 or more categories. (**C**) Clinical outcomes of euploid and mosaic groups. Chr. = chromosome; ICM = inner cell mass; TE = trophectoderm.

Viotti. Mosaic traits affect clinical outcomes. Fertil Steril 2020.

FIGURE 2



Effect of mosaicism level and type on clinical outcomes. (**A**) Analysis of mosaic level (the percentage of aneuploid cells) in 3 groups: all mosaic embryos combined, embryos with whole-chromosome mosaicism, and embryos with mosaicism affecting exclusively segmental abnormalities. Top graphs depict clinical outcomes by mosaic level in 10% increments, with linear regression analysis. The bottom graphs depict clinical outcomes using a cutoff of 50% to differentiate "low" mosaics from "high" mosaics. (**B**) Analysis of clinical outcomes by mosaic type (nature of aneuploidy involved). Chi-square test for trend (blue dotted line and connected points) indicates statistically significant decreasing trend with increasing extent of mosaic aneuploidy. Mos. = mosaic; ns = not significant.

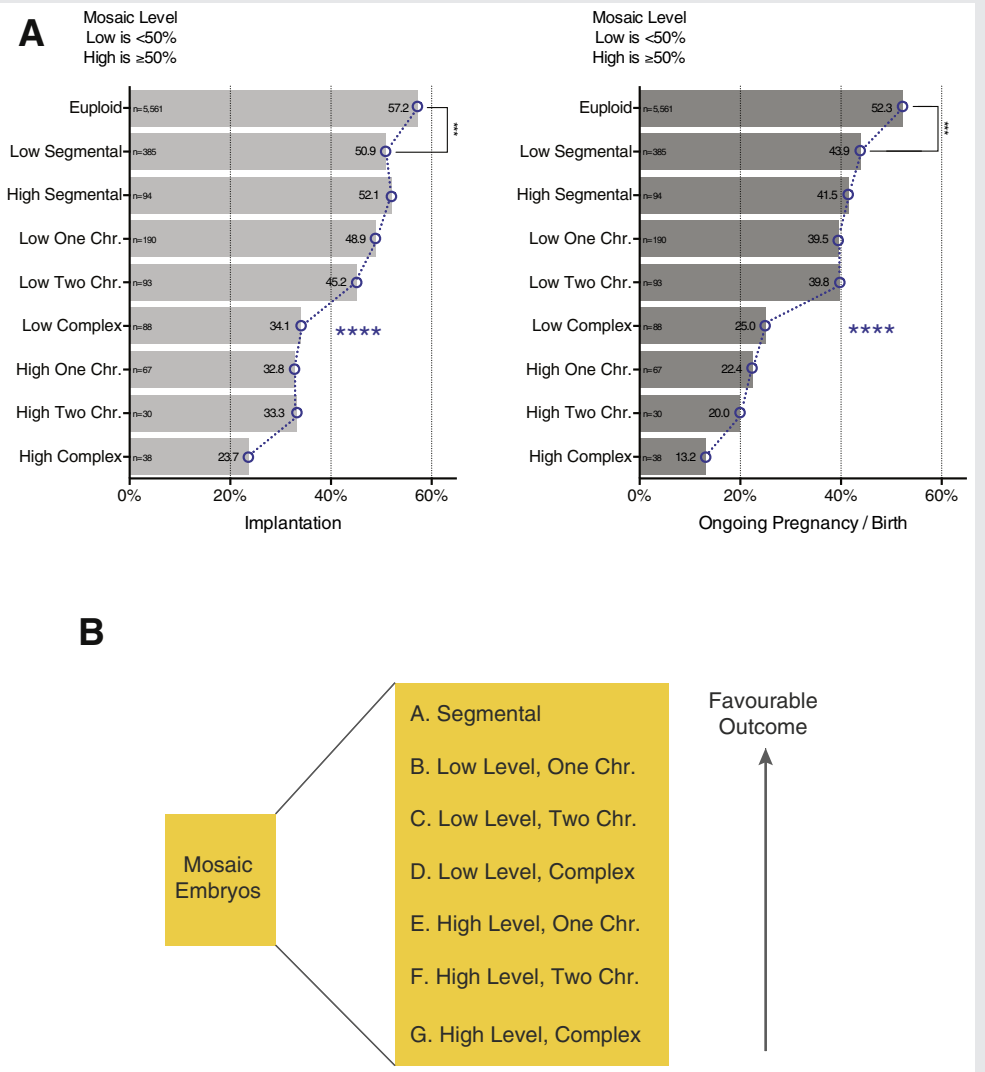
Viotti. Mosaic traits affect clinical outcomes. Fertil Steril 2020.

between the mosaic low and high groups (Fig. 3A and Supplemental Fig. 2, available online). For segmental mosaics, none of the cutoffs yielded significant differences between mosaic low and high groups (Fig. 3A and Supplemental Fig. 2, available online).

Number of Affected Chromosomes in Mosaicism Correlate With Poorer Outcome

We explored whether the type of mosaicism (i.e. the kind of aneuploidy present in the abnormal cells in the mosaic mix)

FIGURE 3



Combined effect of mosaic traits on clinical outcome reveals ranking system for mosaic embryos. (**A**) Clinical outcomes of the euploid group compared with mosaic groups sorted by mosaic level and type. For mosaic level, "low" is <50%, "high" is ≥50%. Chi-square test for trend (blue dotted line and connected points) indicates statistically significant trend. (**B**) Ranking of mosaic embryo subgroups, sorted by favorable clinical outcomes. Chr. = chromosome.

Viotti. Mosaic traits affect clinical outcomes. Fertil Steril 2020.

affected clinical outcomes. First, we considered subchromosomal abnormalities of segmental nature. There were no significant differences in clinical outcomes between embryos with mosaicism affecting a single, two, or more than two segmental regions (Supplemental Fig. 3, available online). However, the combined segmental mosaic group had significantly poorer outcomes compared with the euploid control group (Fig. 3B).

Considering other mosaic types, a chi-square test for trend indicated that clinical outcomes were progressively poorer with increasing severity of mosaic aneuploidy in a statistically significant manner (Fig. 3B). Mosaic segmentals had the best outcomes, followed by the group with one affected whole chromosome, followed by the group with two affected chromosomes, followed by the complex group, in which three or more chromosomes were affected (implantation $P<.0001$; OP/B $P<.0001$). There were no significant differences in outcomes between embryos with mosaic monosomies and trisomies (Fig. 3B).

No Maternal Age Effect on Outcomes of Mosaic Embryos

In the analyzed dataset, maternal age did not affect clinical outcomes. As in the euploid group, mosaic embryos derived from oocytes isolated at a maternal age younger than 34 years

FIGURE 4

Euploid

Euploid	Stage	Implantation	Ong. pregn./birth	ICM	Implantation	Ong. pregn./birth	TE	Implantation	Ong. pregn./birth
	3	0.66	0.70	A	1.00	1.00	A	1.00	1.00
	4	0.87	0.90	B	0.88	0.84	B	0.87	0.86
	5	1.00	1.00	C	0.39	0.33	C	0.50	0.49
	6	0.80	0.77						

Mosaic

Segmental	Stage	Implantation	Ong. pregn./birth	ICM	Implantation	Ong. pregn./birth	TE	Implantation	Ong. pregn./birth
	3	0.53	0.58	A	1.00	1.00	A	1.00	0.93
	4	0.78	0.71	B	0.84	0.74	B	0.76	0.70
	5	0.81	0.79	C	0.24	0.19	C	0.61	0.63

Low level, 1 chr	Stage	Implantation	Ong. pregn./birth	ICM	Implantation	Ong. pregn./birth	TE	Implantation	Ong. pregn./birth
	4	0.69	0.75	A	0.89	0.83	B	0.78	0.70
	5	0.62	0.53	B	0.78	0.64	C	0.52	0.41
				C	0.52	0.40			

Low level, 2 chr	Stage	Implantation	Ong. pregn./birth	ICM	Implantation	Ong. pregn./birth	TE	Implantation	Ong. pregn./birth
	4	0.67	0.73	B	0.85	0.83	A	0.89	0.96
	5	0.86	0.82	C	0.38	0.29	B	0.70	0.70
							C	0.58	0.55

Low level, complex	Stage	Implantation	Ong. pregn./birth	ICM	Implantation	Ong. pregn./birth	TE	Implantation	Ong. pregn./birth
	4	0.89	0.71	B	0.77	0.67	B	0.80	0.58
	5	0.67	0.61	C	0.53	0.22	C	0.57	0.50

High level, 1 chr	Stage	Implantation	Ong. pregn./birth	ICM	Implantation	Ong. pregn./birth	TE	Implantation	Ong. pregn./birth
	4	0.41	0.30	B	0.58	0.36	B	0.51	0.31
	5	0.45	0.39	C	0.48	0.37	C	0.45	0.38

High level, 2 chr	Stage	Implantation	Ong. pregn./birth	ICM	Implantation	Ong. pregn./birth	TE	Implantation	Ong. pregn./birth
	4	0.67	0.49	A	0.42	0.44	B	0.57	0.37
	5	0.49	0.35	B	0.64	0.44			

High level, complex	Stage	Implantation	Ong. pregn./birth	ICM	Implantation	Ong. pregn./birth	TE	Implantation	Ong. pregn./birth
	5	0.47	0.20	B	0.56	0.23	B	0.25	0.13
				C	0.31	0.32			

Matrix of embryo ranking according to mosaicism traits and morphology. Values and cell colors indicate ranking, from best (1.00/*green*) to worst (0.00/*red*). The figure was generated using the data from 5,561 euploid embryos and 1,000 mosaic embryos analyzed in this study, and can serve as a reference to determine prospectively the order of transfer for embryos in the clinic. The combined rank value for an embryo can be assessed by considering its PGT-A (sub-) category and calculating the average of the 3 indicated values (Stage, ICM grade, and TE grade). The resulting number can be compared with that of other embryos in a cohort to establish priority for transfer. A web-based tool of this matrix that performs calculations for the user is available at https://embryo-score.web.app. Chr. = chromosome; ICM = inner cell mass; Ong. pregn. = ongoing pregnancy; PGT-A = preimplantation genetic testing for aneuploidy; TE = trophectoderm.

Viotti. Mosaic traits affect clinical outcomes. Fertil Steril 2020.

had similar rates of implantation and OP/B than those isolated at maternal age of 34 year and older (Supplemental Fig. 4, available online).

Analysis of Mosaicism in Individual Chromosomes

We tabulated the clinical outcomes of all single, whole-chromosome monosomies and trisomies in the 1,000 mosaic

embryo dataset (Supplemental Table 4, available online). There were a total of 157 and 103 embryos with a single mosaic monosomy and trisomy, respectively.

Sample sizes were too small to make relevant "per chromosome" statistical determinations, but the data indicates that blastocyst-stage mosaicism in any of the 23 chromosomes may result in viable pregnancies.

Combining Characteristics of Mosaicism to Determine a Prioritization Scheme for the Clinic

Having observed that 50% was the most significant cutoff to divide embryos into mosaic low and high groups, we analyzed the clinical outcomes of each mosaic type within those two level groups. Statistical analysis indicated the most significant order in which the subgroups correlated with clinical outcomes (Fig. 4A). As noted before, the division into a low and high group was not advantageous for segmental mosaics. For embryos with mosaicism affecting at least one whole chromosome, considering mosaic level and type simultaneously revealed significantly different subgroup outcomes. On the lowest end of the spectrum, the high-level complex mosaic subgroup had the worst outcome rates, but notably those embryos still had some potential to implant and produce ongoing pregnancies and births. Those findings can be summarized in a ranking system of mosaic traits according to clinical outcomes (Fig. 4B).

To consider all relevant parameters for embryo selection in the clinic, we applied an additional analysis of embryo morphology to every subgroup in the ranking scheme. Using the outcomes from the 1,000 mosaic embryo transfers, we tabulated the rates of implantation and OP/B for every permutation of morphology in the Gardner and Schoolcraft system (Supplemental Table 3, available online). Normalizing the data to the best morphological subgroup of the euploid group (5AA) (Materials and Methods), the resulting matrix specified the prioritization order according to mosaic embryo level, type, and stage/grade assessment (Fig. 1). Taking the average of the three indicated values for stage, ICM grade, and TE grade produces a ranking score for any given embryo. Therefore, the table provides a reference to prioritize embryos in the clinic by likelihood of favorable clinical outcome. An interactive web-based tool that utilizes this matrix and performs the relevant calculations can be accessed at https://embryo-score.web.app and is freely available to the user.

DISCUSSION

The present study is the largest dataset of transferred mosaic embryo outcomes reported to date. This compiled analysis conclusively shows that embryos classified as mosaic have a distinct set of clinical outcomes and should comprise a separate PGT-A category. Maintaining PGT-A's "classical" binary system of normal/abnormal is disadvantageous. On the one hand, grouping mosaic embryos with the normal category would result in decreased rates of implantation and increased miscarriages. On the other hand, indiscriminately lumping mosaic embryos with the abnormal category would result in discarding viable embryos. To further increase likelihood of positive clinical outcomes, embryos of the mosaic category should be stratified by their mosaic traits (level and type) identified with PGT-A, and ranked for transfer to a patient. The prioritization scheme outlined in this study can be applied to any embryo by considering its mosaic attributes and morphology.

Recent studies using serial biopsies have shown that uniform euploidy or whole-chromosome aneuploidy are highly concordant between different regions of a blastocyst (22–26). However, mounting evidence indicates that intrabiopsy mosaicism in the TE is a poor predictor of the ploidy status of the remaining embryo, and often pairs with uniform euploidy in the ICM (7, 8, 23, 27). Consequently, our findings suggest that possessing a mix of euploid and aneuploid cells in the TE alone might be sufficient to influence negatively clinical outcomes. Not only were implantation rates lowered, early spontaneous abortions (before week 8–10 of gestation) were particularly frequent with mosaic embryo transfers, possibly indicating the deleterious effects of the aneuploid cell portion in the TE-derived early placenta. That brings up a series of questions: What befalls the aneuploid cells in pregnancies that persist? What happens in cases where mosaicism is present in the ICM as well? And more broadly, how can embryos classified as mosaic using PGT-A produce seemingly healthy births?

There is substantial experimental evidence for corrective mechanisms that operate in the context of chromosomal mosaicism. They center on the well-documented fact that aneuploidy often hampers cell proliferation and viability (28). Studies on murine chimeric embryos formed by mixing cell types showed that euploid cells outcompeted aneuploid cells by differential proliferation and preferential cell death (29, 30). The aneuploid cells replicated more slowly (in the TE) or underwent apoptosis (in the ICM), while euploid cells replicated rapidly and persisted. The experiments revealed a threshold of aneuploid cell load that was incompatible with viability, such that when the ratio of aneuploid to euploid cells was too high at the onset, the embryo invariably died. At mid-to-low ratios, the euploid cells were capable of "rescuing" the embryo by diluting out the aneuploid cells (29). Correspondingly, human mosaic blastocysts subjected to extended in vitro culture frequently displayed a complete loss of the aneuploid cell constituent, and "high" mosaics were more likely to perish during extended culture (31). Live imaging experiments showed that "low"- and "high"-mosaic embryos exhibited significantly different morphokinetics between the zygote and blastocyst stage (32). Furthermore, immunofluorescence analysis of human embryos classified as "euploid" and "mosaic" showed distinct global patterns of cell proliferation and programmed cell death, befitting a model of directed demise of aneuploid cells and compensatory proliferation of euploid cells (7). Our current observations with clinical outcome data build on that concept, suggesting that percent aneuploid cells and the type of aneuploidy together dictate the "severity" of mosaicism. High abnormal cell load and/or complex aneuploidies affecting several chromosomes are more difficult for the embryo to overcome than low abnormal cell load and/or segmental aneuploidies, resulting in distinct implantation and birth rates after transfer.

If aneuploidy can be overcome, should uniformly aneuploid embryos also be considered for transfer? It is important to note that the self-correction mechanisms described apply specifically to mosaicism. When the chromosomal error present in an embryo is the consequence of a meiotic mistake, all of its cells are bound to contain the same aneuploidy. For such an embryo to self-correct and result in a healthy birth, individual cells would need to repair internally their chromosomal abnormalities. Putative mechanisms have been proposed in the literature: "endoreplication" could convert a monosomy into a disomy, or "trisomy rescue" could revert a trisomy into a disomy by anaphase lagging (1). However, these processes frequently would result in uniparental disomy, but uniparental disomy in IVF embryos is extremely rare, estimated at 0.06% (33). Notably, intracellular corrective events have not been documented conclusively in human embryos. In fact, evidence from extended in vitro culture experiments of human embryos suggests the contrary, that uniform euploid or aneuploid embryos invariably maintain their initial ploidy in both ICM and TE lineages (31). Clinical data on this topic is limited, but one study reported the transfer of ten embryos classified as uniform aneuploid using PGT-A, resulting in nine failed pregnancies and a single ongoing pregnancy and birth of an affected baby who died at 6 weeks (5) and a "non-selection study" showed no births from 102 aneuploid embryo transfers (34). The transfer of embryos classified as "aneuploid" using PGT-A, therefore, is not recommended.

Unlike instances of whole-chromosome mosaicism, the embryos composing the segmental mosaic group had uniform outcomes regardless of whether the segmental mosaicism was low or high level, or whether one, two, or more segments were involved in the mosaicism. This observation reflects the unique etiology and repair mechanisms related to segmental abnormalities (35). Unlike whole-chromosome aneuploidies (which mainly arise from chromosome missegregations), segmental deletions and duplications originate from chromosomal breakage via double-strand breaks of the DNA. Double-strand breaks are associated with a distinctive set of corrective pathways, which are dysregulated in early embryogenesis but become more established with developmental progression. Recent studies showed that segmental abnormalities are often discordant between serial biopsies in blastocysts, and are significantly more likely to originate from mitotic errors than from meiotic errors compared with whole-chromosome aneuploidies (22–24, 36). Together, these observations suggest that the postzygotic generation and correction of segmental abnormalities might be more dynamic and reversible than the processes associated with whole-chromosome aneuploidies, possibly explaining why segmental mosaics are better tolerated in regard to implantation and gestation. However, although segmental mosaic embryos as a combined group had better outcomes than whole-chromosome mosaics, they nonetheless had significantly poorer outcomes compared with the euploid control group.

Although there is certainly a need for comprehensive analyses of neonatal outcome data of transferred mosaic embryos, the newborns from our sample group have been invariably healthy based on routine neonatal examination for developmental defects and gross abnormalities. Mosaicism identified using PGT-A at the blastocyst stage is generally not reflected later in gestation during prenatal genetic testing (2, 6, 7), likely because of the aforementioned corrective mechanisms operating in mosaic settings. At present, there is a single report in the literature showing blastocyst-stage mosaicism persisting through gestation, although undergoing a substantial reduction in percent aneuploid cells: from 35% monosomy in chromosome 2 in the TE biopsy at the blastocyst stage to 2% trisomy in chromosome 2 during amniocentesis, in a reciprocal pattern (37). Birth of a healthy baby followed, in which peripheral blood analysis showed 2% mosaic monosomy in chromosome 2, however, epithelial cells in a buccal smear were euploid. Although that neonate had no overt phenotype, this case reinforces the need for careful genetic counselling with emphasis on prenatal testing to patients opting for mosaic embryo transfers (12, 16, 38). This precedent also should be considered when facing the choice between a poor morphology euploid embryo and a good prognosis mosaic embryo. While the ranking matrix presented in this study (Fig. 1) (also available online at https://embryoscore.web.app) unequivocally shows that a low-quality euploid embryo (e.g., ICM grade "C" and TE grade "C") is associated with significantly reduced rates of implantation and OP/B than several mosaic embryo subgroups, the current outstanding questions regarding newborn chromosomal health might nonetheless motivate clinics to favor the transfer of all euploid embryos before resorting to good-quality mosaic embryos. Although newborns resulting from mosaic embryo transfers in this study invariably appeared healthy based on routine examination, concerns for long-term health cannot yet be dispelled entirely. Therefore, the question must be considered carefully by each clinic and patient situation.

Overall, the incidence and variability of the mosaic group at participating centers was in line with previous reports that have used NGS-based PGT-A and the guidelines set forth by Preimplantation Genetic Diagnosis International Society and Controversies in Preconception, Preimplantation and Prenatal Genetic Diagnosis to define "mosaicism." As a reference, the trial 'Single Embryo Transfer of Euploid Embryo' (STAR) reported a proportion of mosaic embryos ranging from 10.5%–26.4% at participating clinics (39). Although the present analysis considered numerous variables that can affect mitotic error rates and clinical outcomes, due to the multicenter nature of the study other potential confounders still persisted (such as differences in demographics, stimulation program, culture techniques, and endometrial preparation methods) and must be noted as a limitation.

The results of the present study contradict the claims that mosaicism is merely an artifact of the PGT-A process. The suggestion that "low"- and "high"-mosaic profiles are respectively euploid and aneuploid profiles with technical noise is discredited by the observation that embryos in the "low"-mosaic category have significantly poorer clinical outcomes than the euploid control group. Conversely, if the "high"-mosaic group was truly no more that uniform aneuploids with technical noise, one would expect virtually no healthy pregnancies from that group. Intrabiopsy mosaicism detected with contemporary, NGS-based PGT-A methodologies,

therefore, is not likely a procedural fluke, but a reflection of a biological occurrence consistent with mosaicism.

CONCLUSIONS

The analysis of 1,000 mosaic embryo transfers provides clinical, statistically significant evidence for the traits of mosaicism identified with PGT-A that affect implantation and spontaneous abortion, offering a blueprint for ranking mosaic embryos in the clinic. The field has been transferring embryos "blindly" for 40 years, and a proportion of those undoubtedly have been of the mosaic category; now refined PGT-A tools can identify and characterize mosaics, allowing for their optimal clinical management.

Acknowledgments: We thank the staff at all participating clinics, laboratories, and centers, without whom this research and analysis could not have been conducted.

REFERENCES

1. Taylor TH, Gitlin SA, Patrick JL, Crain JL, Wilson JM, Griffin DK. The origin, mechanisms, incidence and clinical consequences of chromosomal mosaicism in humans. Hum Reprod Update 2014;20:571–81.
2. Greco E, Minasi MG, Fiorentino F. Healthy babies after intrauterine transfer of mosaic aneuploid blastocysts. N Engl J Med 2015;373:2089–90.
3. Fragouli E, Alfarawati S, Spath K, Babariya D, Tarozzi N, Borini A, et al. Analysis of implantation and ongoing pregnancy rates following the transfer of mosaic diploid-aneuploid blastocysts. Hum Genet 2017;136:805–19.
4. Munné S, Blazek J, Large M, Martinez-Ortiz PA, Nisson H, Liu E, et al. Detailed investigation into the cytogenetic constitution and pregnancy outcome of replacing mosaic blastocysts detected with the use of high-resolution next-generation sequencing. Fertil Steril 2017;108:62–71.e8.
5. Munné S, Spinella F, Grifo J, Zhang J, Beltran MP, Fragouli E, et al. Clinical outcomes after the transfer of blastocysts characterized as mosaic by high resolution Next Generation Sequencing- further insights. Eur J Med Genet 2020;63:103741.
6. Spinella F, Fiorentino F, Biricik A, Bono S, Ruberti A, Cotroneo E, et al. Extent of chromosomal mosaicism influences the clinical outcome of in vitro fertilization treatments. Fertil Steril 2018;109:77–83.
7. Victor AR, Tyndall JC, Brake AJ, Lepkowsky LT, Murphy AE, Griffin DK, et al. One hundred mosaic embryos transferred prospectively in a single clinic: exploring when and why they result in healthy pregnancies. Fertil Steril 2019;111:280–93.
8. Popovic M, Dheedene A, Christodoulou C, Taelman J, Dhaenens L, Van Nieuwerburgh F, et al. Chromosomal mosaicism in human blastocysts: the ultimate challenge of preimplantation genetic testing? Hum Reprod 2018;33:1342–54.
9. Tsuiko O, Zhigalina DI, Jatsenko T, Skryabin NA, Kanbekova OR, Artyukhova VG, et al. Karyotype of the blastocoel fluid demonstrates low concordance with both trophectoderm and inner cell mass. Fertil Steril 2018;109:1127–34.e1.
10. Munné S, Grifo J, Wells D. Mosaicism: "survival of the fittest" versus "no embryo left behind". Fertil Steril 2016;105:1146–9.
11. CoGEN. CoGEN position statement on chromosomal mosaicism detected in preimplantation blastocyst biopsies. Available at: https://ivf-worldwide.com/index.php?option=com_content&view=article&id=733&Itemid=464. Accessed July 26, 2018.
12. Cram DS, Leigh D, Handyside A, Rechitsky L, Xu K, Harton G, et al. PGDIS Position Statement on the Transfer of Mosaic Embryos 2019. Reprod Biomed Online 2019;39(Suppl 1):e1–4.
13. Capalbo A, Ubaldi FM, Rienzi L, Scott R, Treff N. Detecting mosaicism in trophectoderm biopsies: current challenges and future possibilities. Hum Reprod 2017;32:492–8.
14. Van der Aa N, Cheng J, Mateiu L, Zamani Esteki M, Kumar P, Dimitriadou E, et al. Genome-wide copy number profiling of single cells in S-phase reveals DNA-replication domains. Nucleic Acids Res 2013;41:e66.
15. Ramos L, del Rey J, Daina G, Martinez-Passarell O, Rius M, Tunon D, et al. Does the S phase have an impact on the accuracy of comparative genomic hybridization profiles in single fibroblasts and human blastomeres? Fertil Steril 2014;101:488–95.
16. Besser AG, Mounts EL. Counselling considerations for chromosomal mosaicism detected by preimplantation genetic screening. Reprod Biomed Online 2017;34:369–74.
17. Capalbo A, Rienzi L. Mosaicism between trophectoderm and inner cell mass. Fertil Steril 2017;107:1098–106.
18. Murtinger M, Wirleitner B, Schuff M. Scoring of mosaic embryos after preimplantation genetic testing: a rollercoaster ride between fear, hope and embryo wastage. Reprod Biomed Online 2018;37:120–1.
19. Maxwell SM, Colls P, Hodes-Wertz B, McCulloh DH, McCaffrey C, Wells D, et al. Why do euploid embryos miscarry? A case-control study comparing the rate of aneuploidy within presumed euploid embryos that resulted in miscarriage or live birth using next-generation sequencing. Fertil Steril 2016;106:1414–9.e5.
20. Zheng H, Jin H, Liu L, Liu J, Wang WH. Application of next-generation sequencing for 24-chromosome aneuploidy screening of human preimplantation embryos. Mol Cytogenet 2015;8:38.
21. Gardner DK, Schoolcraft WB. In vitro culture of human blastocysts. In: Jansen R, Mortimer D, editors. Towards reproductive certainty: fertility and genetics beyond. Camforth, UK: Parthenon Publishing; 1999:378–88.
22. Girardi L, Serdarogullari M, Patassini C, Poli M, Fabiani M, Caroselli S, et al. Incidence, origin, and predictive model for the detection and clinical management of segmental aneuploidies in human embryos. Am J Hum Genet 2020;106:525–34.
23. Navratil R, Horak J, Hornak M, Kubicek D, Balcova M, Tauwinklova G, et al. Concordance of various chromosomal errors among different parts of the embryo and the value of re-biopsy in embryos with segmental aneuploidies. Mol Hum Reprod 2020;26:269–76.
24. Victor AR, Griffin DK, Brake AJ, Tyndall JC, Murphy AE, Lepkowsky LT, et al. Assessment of aneuploidy concordance between clinical trophectoderm biopsy and blastocyst. Hum Reprod 2019;34:181–92.
25. Huang J, Yan L, Lu S, Zhao N, Qiao J. Re-analysis of aneuploidy blastocysts with an inner cell mass and different regional trophectoderm cells. J Assist Reprod Genet 2017;34:487–93.
26. Lawrenz B, El Khatib I, Linan A, Bayram A, Arnanz A, Chopra R, et al. The clinicians dilemma with mosaicism-an insight from inner cell mass biopsies. Hum Reprod 2019;34:998–1010.
27. Sachdev NM, McCulloh DH, Kramer Y, Keefe D, Grifo JA. The reproducibility of trophectoderm biopsies in euploid, aneuploid, and mosaic embryos using independently verified next-generation sequencing (NGS): a pilot study. J Assist Reprod Genet 2020;37:559–71.
28. Sheltzer JM, Amon A. The aneuploidy paradox: costs and benefits of an incorrect karyotype. Trends Genet 2011;27:446–53.
29. Bolton H, Graham SJ, Van der Aa N, Kumar P, Theunis K, Fernandez Gallardo E, et al. Mouse model of chromosome mosaicism reveals lineage-specific depletion of aneuploid cells and normal developmental potential. Nat Commun 2016;7:11165.
30. Singla S, Iwamoto-Stohl LK, Zhu M, Zernicka-Goetz M. Autophagy-mediated apoptosis eliminates aneuploid cells in a mouse model of chromosome mosaicism. Nat Commun 2020;11:2958.
31. Popovic M, Dhaenens L, Taelman J, Dheedene A, Bialecka M, De Sutter P, et al. Extended in vitro culture of human embryos demonstrates the complex nature of diagnosing chromosomal mosaicism from a single trophectoderm biopsy. Hum Reprod 2019;34:758–69.
32. Lee CI, Chen CH, Huang CC, Cheng EH, Chen HH, Ho ST, et al. Embryo morphokinetics is potentially associated with clinical outcomes of single-embryo transfers in preimplantation genetic testing for aneuploidy cycles. Reprod Biomed Online 2019;39:569–79.
33. Gueye NA, Devkota B, Taylor D, Pfundt R, Scott RT Jr, Treff NR. Uniparental disomy in the human blastocyst is exceedingly rare. Fertil Steril 2014;101:232–6.

34. Tiegs AW, Tao X, Zhan Y, Whitehead C, Kim J, Hanson B, et al. A multicenter, prospective, blinded, nonselection study evaluating the predictive value of an aneuploid diagnosis using a targeted next-generation sequencing-based preimplantation genetic testing for aneuploidy assay and impact of biopsy. Fertil Steril 2020.
35. Viotti M. Preimplantation genetic testing for chromosomal abnormalities: aneuploidy, mosaicism, and structural rearrangements. Genes (Basel) 2020;11:602.
36. Ou Z, Chen Z, Yin M, Deng Y, Liang Y, Wang W, et al. Re-analysis of whole blastocysts after trophectoderm biopsy indicated chromosome aneuploidy. Hum Genomics 2020;14:3.
37. Kahraman S, Cetinkaya M, Yuksel B, Yesil M, Pirkevi Cetinkaya C. The birth of a baby with mosaicism resulting from a known mosaic embryo transfer: a case report. Hum Reprod 2020;35:727–33.
38. Besser AG, McCulloh DH, Grifo JA. What are patients doing with their mosaic embryos? Decision making after genetic counseling. Fertil Steril 2019;111:132–7.e1.
39. Munné S, Kaplan B, Frattarelli JL, Child T, Nakhuda G, Shamma FN, et al. Preimplantation genetic testing for aneuploidy versus morphology as selection criteria for single frozen-thawed embryo transfer in good-prognosis patients: a multicenter randomized clinical trial. Fertil Steril 2019;112:1071–9.e7.