One Hundred Mosaic Embryos Transferred Prospectively In A Single Clinic: Exploring When And Why They Result In Healthy Pregnancies

Running Title: Mosaic embryos and healthy pregnancies

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The authors of this study have nothing to disclose.

Capsule: Chromosomal mosaicism in embryos can exist in a variety of forms, and different characteristics determine the clinical outcome of mosaic embryo transfers.

ABSTRACT

Objective: To investigate the parameters of mosaicism and biological mechanisms leading to healthy pregnancies from mosaic embryo transfers.

Design: Prospective study.

Setting: IVF center and associated research laboratory.

Patient(s): 59 patients.

Interventions: Embryos underwent blastocyst-stage preimplantation genetic testing for aneuploidy (PGT-A) by next-generation sequencing (NGS). Trophectoderm (TE) biopsies containing 20-80% abnormal cells were deemed mosaic, and corresponding blastocysts were transferred. Mosaic embryos donated to research were examined for karyotype concordance in multiple biopsies, and assessed for cell proliferation and death by immunofluorescence and computational quantitation.

Main Outcome Measure(s): Chemical start of pregnancy, implantation, fetal heartbeat, birth.

Results: Globally, mosaic embryos showed inferior clinical outcomes than euploid embryos. Aneuploid cell percentage in TE biopsies did not correlate with outcomes, but type of mosaicism did, as embryos with single mosaic segmental aneuploidies fared better than all other types. Mosaic blastocysts generated from oocytes retrieved at young maternal ages (≤ 34 years) showed better outcomes than those retrieved at older maternal ages. Mosaic embryos displayed low rates of karyotype concordance between multiple biopsies, and showed significant elevation of cell proliferation and death compared to euploid embryos.

Conclusions: After euploid embryos, mosaic embryos can be considered for transfer prioritizing those of the single segmental mosaic type. If a patient has mosaic embryos available that were generated at different ages, preference should be given to those made at younger ages. Intra-blastocyst karyotype discordance and differential cell proliferation and death might be reasons by which embryos classified as mosaic can result in healthy pregnancies and babies.

Keywords: Mosaic; PGT-A; Blastocyst; Aneuploidy; Next-generation sequencing

INTRODUCTION

Chromosomal mosaicism, or the presence of two or more chromosomally distinct cell lines within an individual, has clinical implications both in naturally conceived and IVF pregnancies. Among natural pregnancies it is known to affect ~2% of all gestations in the form of confined placental mosaicism (CPM). This condition entails discordance of karyotypes between fetal and placental cells and can lead to adverse obstetric outcomes including intrauterine growth retardation or placental insufficiency (1, 2). Among IVF embryos, data from a flurry of recent studies suggests that, in general, mosaicism results in decreased pregnancy rates compared to normal embryos (3-7). However, numerous forms of mosaicism exist, and refinement of these interpretations is needed. The contemporary IVF clinic must grapple with the question: What should be done with mosaic embryos, should they be transferred, and if so, how should they be prioritized?

Preimplantation genetic testing for aneuploidy (PGT-A) at the blastocyst stage is currently used in over 20% of all IVF treatments in the USA, and growing (3). It entails analysis of the chromosomal content of a representative 5-10 cell biopsy taken from the trophectoderm (TE) tissue and produces a readout estimating the copy number of each chromosome. For autosomes, a copy number two is indicative of a disomy (considered normal/euploid), while copy numbers of one and three are indicative monosomy and trisomy, respectively (considered abnormal/aneuploid). In such cases, the clinical decision to de-select aneuploid embryos for transfer is straightforward. A third classification category exists, namely samples with analysis

readouts producing values at intermediate levels between whole numbers. Such profiles are consistent with mosaicism, which would indicate the presence of both euploid and aneuploid cells in the source blastocyst. While previous technologies for PGT-A were limited in identifying this condition, next-generation sequencing (NGS) is now widely recognized as the most accurate platform for revealing and quantifying mosaicism (8).

In order to better define the characteristics and genetic abnormalities affecting the clinical outcomes of mosaic embryos, we performed an analysis of the prospective transfer of 100 embryos classified as mosaic via NGS-based PGT-A in a single IVF center. Furthermore, we explore biological mechanisms that can lead a mosaic blastocyst to ultimately result in a healthy baby.

MATERIALS AND METHODS

Patients and Embryos

Embryos derived from patients seeking infertility treatment at a private IVF center were generated by intracytoplasmic sperm injection (ICSI) and cultured to the blastocyst stage as previously described (9). Blastocysts were assessed with the Gardner evaluation system (10) and subjected to a 5-10 cell TE biopsy and vitrified until further use. Biopsies were processed for PGT-A (see details below). In cases where no euploid blastocysts were available, patients were counseled about the possibility of selecting blastocysts classified as mosaic for uterine transfer. All embryos described in this study were transferred in a prospective manner, meaning that prior knowledge of the mosaic status of the embryos was available in every case. In certain instances, more than one mosaic blastocyst was transferred at once, or one mosaic blastocyst was transferred along with a euploid blastocyst (generally of poorer quality), especially in patients with previous failed transfers.

Clinical outcomes were defined and collected as follows: Beta human Chorionic Gonadotropin (Beta-hCG) was measured by blood test on day 10 after transfer, with values > 5.0 mIU/mL considered positive and indicative of start of pregnancy. Presence of a gestational sac observed by endovaginal ultrasound at 3-5 weeks after transfer was considered evidence of implantation. Fetal heartbeat (FHB) was confirmed by endovaginal ultrasound 6-8 weeks after transfer. Non-Invasive Prenatal Testing (NIPT), amniocentesis, and birth information were voluntarily reported by the patient.

The experiments of serial biopsy concordance and cell proliferation and death made use of supernumerary embryos donated to research by informed consent.

This study was approved by the Zouves Foundation IRB (OHRP IRB00011505).

PGT-A

NGS-based PGT-A was performed in-house using VeriSeq kit (Illumina) on a MiSeq system (Illumina) following the manufacturer's protocol, in 24 sample runs. Karyotype profiles were scored independently by two analysts using Bluefuse Multi Analysis Software (Illumina), which depicts the copy number for each chromosome in a sample. The platform is validated to detect segmental gains/losses of 20 Mb or larger by the manufacturer, but can occasionally detect regions smaller than 2 Mb (11). A molecular karyotype profile consistent with mosaicism was determined when a whole chromosome or sub-chromosomal segment resulted in intermediate copy number levels (in the range of 20-80% between whole numbers), following PGDIS guidelines (http://www.pgdis.org/docs/newsletter_071816.html) and as previously described (6).

Mosaic Study of Cell and DNA Mixes

For cell analysis, the following cell lines were used: Coriell GM00425 (+8) and GM04435 (+16, +21). Cells were cultured in RPMI 1640 (Thermo Fisher #12633-012) containing 10% FBS (Seradigm 1500-050), GlutaMAX-I (Gibco 35050-061) and Pen-Strep (Gibco 15140-122). Cells were detached with TrypLE (Gibco 12604021) and re-suspended in culture medium. Single cells were collected and mixed in the indicated ratios totaling 10 cells per sample and stored at -80°C until chromosomal

analysis by NGS as above. Each cell ratio was performed in triplicate, and one representative karyotype profile is shown per tested ratio in the Fig. 1A.

For DNA analysis, we obtained genomic DNA extracted from two aneuploid fibroblast cell lines. The first DNA sample (Coriell NA02948) was purified from cells trisomic for chr13. The second DNA sample (Coriell NA00072) was purified from a cell line advertised as containing a segmental loss in chr4p. We found additional chromosomal errors (-13, mos(-5q,-11p,-12p,+17q)) in the sub-clonal line used in this study (lot 1 with original extraction date 4/28/1997) and verified them in over 40 test runs. In the singlicate mixing experiments, DNA was diluted down to represent equivalent amounts contained in single diploid cells (6.6pg), such that a 50:50 mix of DNAs contained 33pg of DNA from each cell line for a total of 66pg, equivalent to DNA from 10 diploid cells per NGS reaction.

Multiple Biopsy Experiment

Mosaic blastocysts as determined from the original clinical TE biopsy were further processed to isolate an ICM biopsy and a second TE biopsy as described elsewhere (12). All biopsies underwent PGT-A as described above. DNA fingerprinting using a previously described method (12) was performed on every biopsy to confirm it originated from its intended blastocyst, thereby excluding the possibility of sample mislabeling or contamination.

Immunofluorescence

Blastocysts were immersed in fixation buffer containing 4% paraformaldehyde (EMS #15710) and 10% fetal bovine serum (FBS) (Seradigm 1500-050) in phosphate buffered saline (PBS) (Corning MT21040CM) for 10 minutes (min) at room temperature (rt), followed by three 1 min washes at rt in stain buffer composed of 0.1% Triton X-100 (TX-100) (Sigma X100-100ML) and 10% FBS in PBS. Samples were then immersed in permeabilization buffer (0.5% TX-100, 10% FBS in PBS) for 30 min at rt, followed by three washes in stain buffer. Samples were then exposed to stain buffer containing both primary antibodies (abs) each in 1:200 concentrations over night at 4°C rocking on a nutator. Primary abs were rabbit anti-human phospho-Histone H3 (Ser10) (pHH3) AlexaFluor555 conjugated monoclonal ab (Cell Signaling #3475), rabbit anti-human Cleaved Caspase-3 (Asp175) AlexaFluor647 conjugated monoclonal ab (Cell Signaling #9602), and mouse anti-human OCT-3/4 monoclonal ab (Santa Cruz sc-5279). The next day, after three washes in stain buffer, samples were immersed in stain buffer containing the secondary antibody goat anti-mouse IgG AlexaFluor488 (Thermo Fisher A11029) at 1:500 concentrations for 2-3 hours at rt. After three washes in stain buffer, samples were exposed to nuclear stain (Hoechst 33342, Thermo Fisher H3570) diluted at 1:1000 in stain buffer for 30 min at rt, followed by three more washes in stain buffer and subsequently imaged.

Imaging and Computational Quantitation of Cell Proliferation and Death

Stained blastocysts were placed in glass bottom dishes (MatTek P35G-1.5-20-C) in small drops of stain buffer overlaid with mineral oil (Sigma M5904), and imaged with a LSM 780 Confocal microscope (Zeiss). Image files in the .lsm format were

uploaded into the software package Imaris 8.4.1 (Bitplane), and fluorescent channels quantified for each blastocyst. The analysis was performed in a blinded fashion, as all samples were quantified computationally with a uniform set of parameters, independently of blastocyst classification. The parameters were:
Nuclear channel: spots with estimated diameter = 8.00um, background subtraction = true, classify spots by quality above 13.6. OCT3/4 channel: spots with estimated diameter = 6.00um, background subtraction = true, classify spots by quality above 11.0. pHH3 channel: spots with estimated diameter = 8.00um, background subtraction = true, classify spots by quality above 15.5. Caspase-3 channel: surfaces with enable smooth = true, surface grain size = 0.700um, enable eliminate background = true, diameter of largest sphere = 8.00um, manual threshold value = 7.64, active threshold B = false, classify surfaces by number of voxels above 204.

Statistics

Analysis and graph preparation was done in Prism 6 (GraphPad). In Table 2, clinical outcome comparisons between groups (defined in the table footnotes) were performed with Fisher's exact test. Note that for the analyses in Table 2, for double embryo transfer in which only one embryo was positive but its identity could not be resolved due to matching sexes, each embryo received a value of 0.5. When this scenario occurred in cases of triple embryo transfers, each embryo received a value of 0.33. Final numbers are shown rounded to the closest integer.
In the mitosis/apoptosis quantitation experiment (Figure 1), differences between groups were assessed by unpaired, two-tailed Student's t test with Welch's

correction. For all analyses: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns (not significant), $P \geq 0.05$.

RESULTS

Detection of chromosomal mosaicism with NGS-based PGT-A

Previous reports elegantly demonstrated that mixes of cell lines with different karyotypes resulted in mosaic profiles during PGT-A (3, 6, 13, 14), and that NGS showed superior resolution and more accurate mosaic calling than other platforms, including array comprehensive chromosome hybridization (aCGH) (13). We sought to use similar spike-in experiments to confirm the ability to detect chromosomal mosaicism in our hands, using an in-house PGT-A platform. As a first pass test, we observed that mixes of individual cells from lines with different aneuploidies yielded expected profiles consistent with mosaicism (Figure 1A). Then, in order to better establish the resolution of the technology in detecting mosaicism we performed experiments with DNA purified from cell lines. This allowed for more refined mixing ratios than with whole cells. We took advantage of cell line-derived DNA that displayed a very distinct karyotypic profile: copy number 2 for some parts of the genome (consistent with disomy), copy number 1 for other regions (consistent with monosomy), and intermediate levels (consistent with mosaicism) for yet other regions (Figure 2B, See DNA Cell Line B). This particular profile was replicated in over 40 sequencing runs, suggesting that at the time of DNA purification the cell line was not uniform and contained sub-clones resulting in mosaic profiles in some genomic regions. Mixing experiments with DNA from a different, uniformly aneuploid cell line showed superb resolution of mosaic profiles, with resolution of differences as small

as ~5% (Figure 2B). This was true for both whole chromosome and segmental (sub-chromosomal) aneuploidies.

Together, these experiments confirmed the capability to detect instances of chromosomal and segmental mosaicism with our in-house NGS-based PGT-A pipeline.

Clinical outcome of prospective mosaic embryo transfers tested by NGS-based PGT-A

Between October 2016 and June 2018, a total of 100 blastocysts classified as mosaic by NGS-based PGT-A were transferred in a prospective manner (with previous knowledge of their mosaic status) at a single IVF program as frozen embryo transfers (Table 1). 50 were replaced into patients as single embryo transfers (SET), 26 as double embryo transfers (DET), and 6 as triple embryo transfers (TET). The remaining 18 were transferred alongside one euploid embryo often of poor morphological quality (Supplemental Table 1).

Compared to euploid blastocysts across all medical indications and ages transferred in the same time period, the combined mosaic cohort had significantly lower implantation rates per embryo (as determined by the presence of a gestational sac)(49.6% vs 38.0%)(Table 2). Mosaic embryos also resulted in significantly lower chances of developing a fetal heartbeat (FHB)(47.1% vs 30.0%)(Table 2). Patients who had only a single euploid blastocyst available for transfer (without the possibility of further embryo selection) experienced clinical outcomes that were inferior to the

combined group of euploid blastocysts, but were appreciably superior to the mosaic blastocyst cohort (45.0% implantation, 42.2% FHB) (Table 2).

Of the 30 mosaic embryos resulting in FHB in this study, 11 were ongoing pregnancies at the time of manuscript preparation and the remaining 19 have resulted in births. One patient who had two mosaic embryos transferred simultaneously went into labor at 23 weeks with spontaneous rupture of membranes (SRM) leading to death of both newborns, which showed no other physiological abnormalities upon examination.

In 7 cases where NIPT information could be retrieved from patients, all results were normal. Amniocentesis was performed and data retrieved in 11 cases, 8 of which tested normal. One case contained a balanced translocation, and two cases showed microdeletions affecting segments smaller than the validated resolution of the PGT-A platform used (Table 1).

Hence, the combined cohort of mosaic embryos showed overall decreased implantation rates compared to euploids. Of the 30 cases showing a FHB there were no instances of clinical miscarriage to date.

Parameters of mosaicism affecting clinical outcome

In our dataset, blastocysts showing mosaicism exclusively in a single segmental (sub-chromosomal) region resulted in considerably better clinical outcomes than blastocysts with other types of mosaicism (i.e. affecting multiple segments or any number of whole chromosomes) (Table 2).

The degree of mosaicism, which is an estimate of the percentage of aneuploid cells in the TE biopsy, did not correlate with clinical outcome in our dataset. We came to the same conclusion when analyzing the data using two different cutoffs for 'low' versus 'high' degree of mosaicism, and the differences between groups were statistically insignificant (Table 2).

Interestingly, we observed a significant age effect on the success of mosaic embryo transfers. When oocytes were retrieved from patients 34 years old or younger, the clinical outcomes of their resulting mosaic blastocysts were considerably better than those retrieved from patients greater than 34 years old (Table 2). This was true for all types of mosaicism (Supplemental Table 2). In the control group, euploid blastocysts fared equally well regardless of age.

Finally, we saw no difference in rates of implantation or FHB when the mosaicism affected chromosomal gains (trisomies) versus losses (monosomies) (Table 2).

Mosaicism in the clinical TE biopsy is a poor predictor of chromosomal content in the remaining blastocyst

We hypothesized that some mosaic blastocysts might lead to normal pregnancies because the clinical TE biopsy collected might not be representative of the entirety of the blastocyst and particularly the ICM, which could be euploid. To test this experimentally, we took an ICM biopsy and an additional TE biopsy from five blastocysts that were originally classified as mosaic (Table 2). In three blastocysts, the ICM biopsy was euploid, while in two blastocysts the ICM biopsy displayed

mosaicism that was reciprocal to that observed in the clinical TE biopsy. The reciprocal patterns suggested incidences of chromosomal non-disjunction as the root cause of mitotic error resulting in mosaicism (2). It has been suggested that a reciprocal gain/loss in different biopsies of the same blastocyst is the strongest evidence of true mosaicism (15). In regards to the second TE biopsies collected, such reciprocal patterns were observed in three of the five blastocysts, while the two other samples were euploid.

We performed the same multi-biopsy experiment on three blastocysts with uniform aneuploidies as well as mosaic aneuploidies within their clinical TE biopsies (Table 2). The uniform aneuploidies were always present in the subsequent biopsies for all three embryos. The mosaic aneuploidies observed in the clinical TE biopsy were replicated in subsequent biopsies in only one blastocyst (see Blastocyst 8, Table 2). For that case, the degree of mosaicism varied greatly between biopsies (30% in the clinical TE biopsy, versus 65% in the ICM and 50% in the second TE biopsy).

We performed DNA fingerprinting on all sequenced biopsies to confirm there were no sample mix-ups or contamination (Supplemental Figure 1). Notwithstanding the limited sample size, this experiment demonstrated that embryos classified as mosaic by PGT-A can be euploid in other regions of the blastocyst including the ICM, and that the degree of mosaicism observed in the clinical TE biopsy are not strongly correlated with the degree of mosaicism in subsequent biopsies.

Cell proliferation and death rates are elevated in mosaic blastocysts compared to euploid blastocysts

It has been suggested that euploid cells of mosaic embryos outcompete aneuploid cells during development, possibly leading to chromosomally normal babies. Experimental evidence for such a mechanism comes from a mouse model, in which chimeras of euploid cells and aneuploid cells showed selective apoptosis of aneuploid cells in the ICM and proliferative defects in aneuploid cells of the TE, leading to a progressive depletion of aneuploid cells from the blastocyst stage onwards (16).

If such a model were to apply in the context of human blastocysts generated by IVF, we reasoned that mosaic embryos might display different patterns of cell proliferation and death compared to euploid embryos. In order to test this hypothesis, we performed immunofluorescence experiments on human embryos with markers of mitosis (cell proliferation) and apoptosis (programmed cell death). Serine 10 on histone H3 becomes phosphorylated specifically during mitotic chromatic condensation, making phosphohistone 3 (pHH3) an oft-used marker of mitotic activity. Caspase-3 is the central executioner of apoptosis, and its active (cleaved) form is a validated marker of apoptotic cells. In addition, we stained all blastocysts in this experiment with the nuclear dye Hoechst in order to visualize the nuclei of all cells, as well as OCT-3/4 to be able to differentiate ICM cells from TE cells. After staining euploid, mosaic, and aneuploid blastocysts, they were visualized by a confocal microscope, and fluorescent signals were quantified computationally (Figure 2A and Supplemental Video 1).

In general, levels of mitosis were relatively low in euploid blastocysts (Figure 2B); even though no actively dividing ICM cells were detected in all five euploid blastocysts used in our experiment, these findings do not suggest that cell proliferation does not occur in the ICM of euploid blastocysts (which would be impossible). Instead, it is important to note that immunofluorescent staining produces a static snapshot of development, and rates or levels of cell proliferation and death should be analyzed in a comparative fashion with other groups (i.e. mosaic and aneuploid). Euploid blastocysts displayed negligible levels of apoptosis in the TE as well as in the ICM (Figure 2B), agreeing with previous observations made in normal human blastocysts (17, 18).

Interestingly, a large proportion of mosaic as well as aneuploid blastocysts displayed medium or high levels of mitosis and apoptosis in the TE compared to euploid blastocysts (Figure 2B). In the ICM, some mosaic and aneuploid blastocysts displayed minimal/low levels of mitosis and apoptosis, and others displayed medium or high levels of mitosis and apoptosis (Figure 2B). Together, this data suggest that levels of cell proliferation and death are considerably higher in mosaic and aneuploid blastocysts when compared to euploid blastocysts.

DISCUSSION

PGT-A has undergone numerous technical advances since its inception. Compared to its initial form using FISH, which was limited to relatively few chromosomes, the most recent incarnation based on NGS permits the analysis of all chromosomes as well as detection of chromosomal mosaicism. What is more, mosaics can be further subdivided into categories by degree of mosaicism (low and high) and mosaic type (single or multiple segmental mosaics, whole chromosome mosaics, complex mosaics). This has undoubtedly added layers of complexity to the clinical interpretation of PGT-A results, and evidence-based guidelines are needed.

Preimplantation Genetic Diagnosis International Society (PGDIS) (19) and Controversies in Preconception, Preimplantation, and Prenatal Genetic Diagnosis (CoGEN) (20) provide position statements concerning prioritization of mosaic embryos for transfer, but the rationale behind them remains mainly theoretical. An alternative set of recommendations have been proposed, based on risk levels deduced from mosaic patterns observed in chorionic villus sampling (CVS) of the placenta and in products of conception (POC) (21). Nonetheless, a direct link between types of mosaicism at the blastocyst stage and clinical outcomes will only become defined over time with studies such as this one.

To date, all studies comparing euploid and mosaic embryo outcomes in IVF concur that embryos classified as mosaic can lead to babies that are healthy by routine examination, but with decreased success rates compared to euploids (3-7). Nonetheless, there is some disagreement between studies about which mosaic

parameters correlate with clinical outcome. For example, some have suggested that a high degree of mosaicism (i.e. high proportion of aneuploid cells in the TE biopsy) correlates with poorer outcomes. This relationship was described in a paper using a mix of PGT-A technologies to detect mosaicism (7) as well as a study analyzing NGS data in a retrospective manner, although in the latter report the trend was not statistically significant (6). To our knowledge, this is the first prospective study relying entirely on NGS, widely recognized as the most precise method to detect mosaicism in PGT-A (8, 13, 22). Our data suggests that the degree of mosaicism should not be used to prioritize mosaic embryos. This is contrary to current guidelines expressed by PGDIS or CoGEN to prioritize selection of embryos for transfer, which in our opinion should be amended.

This interesting point warrants discussion from a conceptual standpoint. Few would dispute the notion that a mosaic blastocyst with a high percentage of aneuploid cells is less likely to succeed than one with low percentage of aneuploid cells, a concept first explored in an extensive manner by Verlinsky and colleagues (23) and convincingly demonstrated experimentally in a mouse model of chimeric blastocysts (16). It follows logically that if a clinical TE biopsy were a good representative of the proportion of aneuploid cells in the remaining blastocyst, embryos with high mosaicism in the TE biopsy should fare poorly. The salient point shown in our data is that mosaicism in the TE biopsy is a poor representative of the blastocyst. Mosaic blastocysts do not distribute aneuploid cells evenly, meaning there is an inherent sampling error when collecting the TE biopsy. Therefore, we conclude that the degree of mosaicism in the TE biopsy might be irrelevant to clinical outcome.

One parameter with a substantial effect on clinical outcome in our study was type of mosaicism: single segmental mosaics fared better than all other types, namely those affecting multiple segmental gains/losses, 1 or 2 whole chromosomes, or complex mosaics. This observation agreed with a previous retrospective study (3), although in our dataset the single segmental mosaic embryos did not fare quite as well as euploid embryos. It has been suggested that the better clinical outcomes in segmental mosaics might be due to the fact that segmental aneuploidies typically result from DNA double strand break events, which often activate checkpoint processes leading to cell cycle arrest or apoptosis (3). As a result, neighboring euploid cells could quickly and efficiently dilute out cells containing segmental gains or losses. Also, segmental aneuploidies resulting in acentric fragments do not contain a centromere and cannot attach to the spindle during mitosis, potentially leading to their loss during cell division (3). Therefore, our data supports the notion of prioritizing single segmental mosaics for transfer above other mosaic types.

Another parameter to show a significant effect was age. Mosaic blastocysts derived from oocytes retrieved from patients 34 years old or younger fared significantly better than when derived from older patients. Interestingly, the ‘young’ mosaic group yielded comparable results to euploid embryos, which in itself did not display an age effect upon transfer. We can only speculate the biological reason for this. Could any of the self-correcting mechanisms that have been proposed in mosaic embryos (clonal depletion, preferential allocation, cell-endogenous rescue, see (24)) be more efficient in ‘younger’ blastocysts? It has been documented that, as opposed to meiotic errors and uniform aneuploidy, rates of mitotic error and consequently

mosaicism at the blastocyst stage remain relatively constant with increasing age (2, 25). It is possible that ‘younger’ blastocysts manage to purge themselves of mosaicism, while older blastocysts more often retain their aneuploid cell load and accordingly become less likely to implant and reach birth. Another possibility is that an increasing proportion of mosaic blastocysts generated from older patients originated from trisomy rescue of uniformly aneuploid embryos, in turn possibly leading to negative outcomes. These concepts warrant further investigation and to our knowledge age has not been considered or analyzed as a mosaic parameter in previous studies. If confirmed, in cases where a patient has multiple mosaic blastocysts to choose from that were generated from different cycles at different ages, we would recommend prioritizing the ‘younger’ ones.

We explored two concepts that might explain why embryos classified as mosaic in PGT-A might lead to ongoing pregnancies and healthy births. The first, as mentioned above, is that mosaicism in the TE biopsy is not a good predictor of karyotype elsewhere in the blastocyst. We observed examples where the corresponding ICM as well as a second TE biopsy were euploid. Other cases had reciprocal mosaic aneuploidies in subsequent biopsies. In yet another scenario, a blastocyst had the same mosaic aneuploidy in all three biopsies analyzed, but the degree of mosaicism was different (‘low’ in the clinical TE biopsy, and ‘high’ in the ICM and second TE biopsies The inherent degree of sampling error in isolating a biopsy from a mosaic blastocyst imposes a ‘biological’ source of false positive/negative calls for mosaicism in PGT-A. Ultimately, this poor predictive power of a mosaic TE biopsy vis-a-vis the remaining embryo might explain why embryos

classified as mosaic do occasionally implant and lead to healthy births but do so with lower success rates than euploid embryos. Sometimes the mosaic TE biopsy will pair with euploidy, other times with mosaicism, and yet other times with aneuploidy in the remaining blastocyst. This is not to say that a mosaic TE biopsy will correspond in equal rates to euploidy, mosaicism, and aneuploidy elsewhere. Only a larger and detailed investigation analyzing serial biopsies in embryos classified as mosaic will shed light into such ratios.

It must be acknowledged that there also exists an inherent risk for technical error during PGT-A, which could produce profiles appearing mosaic when in fact the biopsy in question is uniformly euploid or aneuploid. The mixing experiments suggest that NGS-based PGT-A is excellent at identifying mosaicism when indeed present (manifested as intermediate levels of karyotype profiles), but the inverse it not necessarily true: an intermediate karyotype profile does not automatically mean that a TE biopsy contains mosaicism. Artifacts introduced during WGA or NGS could result in background noise that can produce such intermediate levels as well, falsely resulting in karyotype profiles interpretable as mosaic. Our cell mixing experiments, which were performed in biological triplicates, showed a false positive rate for mosaicism of 0% but the sample size was small and there are aspects of TE biopsy (resulting from laser use, biopsy isolation, handling etc.) that cannot be properly modeled in cell mixes. Hence, it has been proposed that rather than categorically diagnosing blastocysts as ‘mosaic’, PGT-A results should indicate a pattern ‘consistent with possible mosaicism’ (15).

The second concept we explored that could make mosaic blastocysts result in healthy births is self-correction. It is known that the incidence of mosaicism decreases through development (6), which could be explained by the out-competition of aneuploid cells by euploid cells by differential cell proliferation and death. Indeed, a chimeric mouse model for mosaicism has shown the progressive depletion of aneuploid cells in the preimplantation embryo (16). In those experiments, aneuploid cells in the fetal lineage (ICM) were largely eliminated by apoptosis, whereas those in the placental lineage (TE) displayed severe proliferative defects. Our findings confirm that in the human embryo, the dynamics of cell proliferation and death are different, on average, between euploid, mosaic, and aneuploid blastocysts. This could correspond to the proposed self-correction mechanism, as aneuploid cells might proliferate slower or undergo apoptosis, and euploid cells compensate by elevating their rates of proliferation. Unfortunately, existing tools and reagents do not allow us to individually visualize the aneuploid and euploid cells in a mosaic human embryo, which would be required to confirm this model. Yet, and notwithstanding the limited sample size of our experiment, analysis on the blastocyst level showed statistically significant differences between groups. Importantly, not all blastocysts classified as mosaic had elevated rates of cell proliferation and death; some showed similar levels to euploids. Presumably, those could be instances of blastocysts with mosaicism in the TE biopsy, but euploidy elsewhere.

Centers using PGT-A have reported vastly different incidences of mosaic embryos, anywhere from less than 4% up to 90% (15). Without context, such comparisons are virtually meaningless. Equal thresholds, cutoffs, and technological

platforms need to be employed to make reasonable comparisons between groups. Regardless of methods used to identify mosaics, the existence of chromosomally mosaic embryos is an undisputed biological phenomenon. In our center, 18% of blastocysts analyzed by PGT-A (n=3138) are classified as ‘mosaic’ using the methods described in the manuscript. This is consistent with the 21% figure reported by a large reference lab using the same standards as described here (22). While biological and technical false positive/negative rates for mosaicism in PGT-A are being established, a preponderance of evidence now shows that the ‘mosaic’ category of blastocysts contains its own distinct set of clinical outcomes, different to the uniform euploid or aneuploid categories. Considering the importance of the mosaic group, evidence-based guidelines are vital to help prioritize them for transfer.

In summary, our findings suggest that after euploids, embryos displaying single mosaic segmental gains and losses should be prioritized for transfer, along with mosaic blastocysts derived from oocytes retrieved at younger patient age. On the other hand, degree of mosaicism in the TE biopsy is not a relevant factor, and blastocysts harboring mosaic monosomies and trisomies result in similar clinical outcomes. Even though to our knowledge this is the largest single-center study of its kind to date, we note that the sample size is still relatively limited and future larger studies will need to corroborate or refute our findings. Finally, we provide experimental data for two possibly parallel/additive mechanisms that may explain why mosaic blastocysts can result in healthy babies, which has been a great concern when transferring embryos classified as mosaic by PGT-A.

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TABLE LEGENDS

Table 1. List of mosaic blastocyst transfers

[1] Estimated percentage of aneuploid cells in biopsy. When several chromosomes affected, highest value is indicated.

* Only applies to one embryo in the double FET, due to equal genders it cannot be deduced which one.

Only applies to one embryo in the triple FET, due to equal genders it cannot be deduced which one.

[a] Single ventricle congenital heart defect detected at 22 weeks, NIPT was normal for all whole chromosomes and microdeletions tested, including DiGeorge deletion (22q11.2).

[b] Microdeletion of one copy comprising 84.11 Kb at 2q13.

[c] Balanced translocation of 1p and 16p.

[d] Microdeletion of one copy less than 100 Kb (no further information available)

[e] Spontaneous rupture of membranes (SRM) at 23 weeks leading to neonatal death of both babies, which showed no other physiological abnormalities upon examination

Table 2.

Analysis of mosaic parameters affecting clinical outcomes

[a] Compared to the 'Euploid All' group.

[b] Compared to previous row (intra-group comparison)

[c] Multi-biopsy analysis. Square brackets indicate the estimated degree of mosaicism observed in the karyotype profile.

Supplemental Table 1. List of double embryo transfers using one mosaic and one euploid blastocyst

Supplemental Table 2. Age of oocyte at retrieval affects clinical outcome in all types of mosaic embryo transfers

FIGURE LEGENDS

Figure 1.

Validation of PGT-A method in accurate identification of mosaicism.

(A) Results from cell mixtures using a total of 10 cells per reaction.

(B) Composite image from spike-in experiments with varying ratios of purified DNA from two aneuploid cell lines. Amounts of DNA mimic contents of single cells (6.6pg), resulting in 66pg per reaction. Results depict karyotype patterns consistent with the expected presence of mosaicism using NGS-based PGT-A. Note for example that the aneuploid region on chr5 is at ~50% loss when solely using DNA from cell line B, suggesting that each incremental mix with DNA from cell line A translates into a ~5% difference.

Figure 2.

Quantitation of cell proliferation and death in blastocysts.

(A) Left column shows representative immunofluorescent images of a hatching blastocyst classified as mosaic by PGT-A. Right column shows method of computational detection and quantitation. Note

that the image analysis software detects the concrete number (count) of nuclei, ICM cells, and cells in mitosis. Apoptosis is measured as arbitrary units (AU) of fluorescence in regions displaying the signal in order to capture all apoptotic bodies, including remaining vesicles of fractioned cells. Scale bar = 20μm

(B) Scatter dot plots depicting quantitation of mitosis and apoptosis in TE and ICM. Each symbol represents one blastocyst. Lines indicate mean with standard deviation. Sample size of each blastocyst group is n=5 Euploids, n=11 Mosaics, n=14 Aneuploids. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns (not significant), $P \geq 0.05$.

Supplemental Figure 1.

Analysis of tissue relatedness in serial biopsies of blastocysts

Graph depicting log-likelihood ratios of relatedness. In green, controls comparing biopsies from embryos derived from unrelated patients, showing negative values. In red, control comparison between biopsies from blastocysts derived from the same patient (full-sibs) showing positive values. In purple, comparisons between paired biopsies for each blastocyst analyzed in the study, showing positive log-likelihood ratios of relatedness.

VIDEO LEGENDS

Supplemental Video 1. Video of confocal microscopy-imaged embryo after immunofluorescence staining, displaying quantitation method. This representative sample was classified as mosaic with PGT-A. Note that the image analysis software detects the concrete number (count) of nuclei (blue), ICM cells (green), and cells in mitosis (red). Apoptotic signal (white) is a measure of volume in regions displaying the signal in order to capture all apoptotic bodies, including remaining vesicles of fractioned cells.

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Figure1

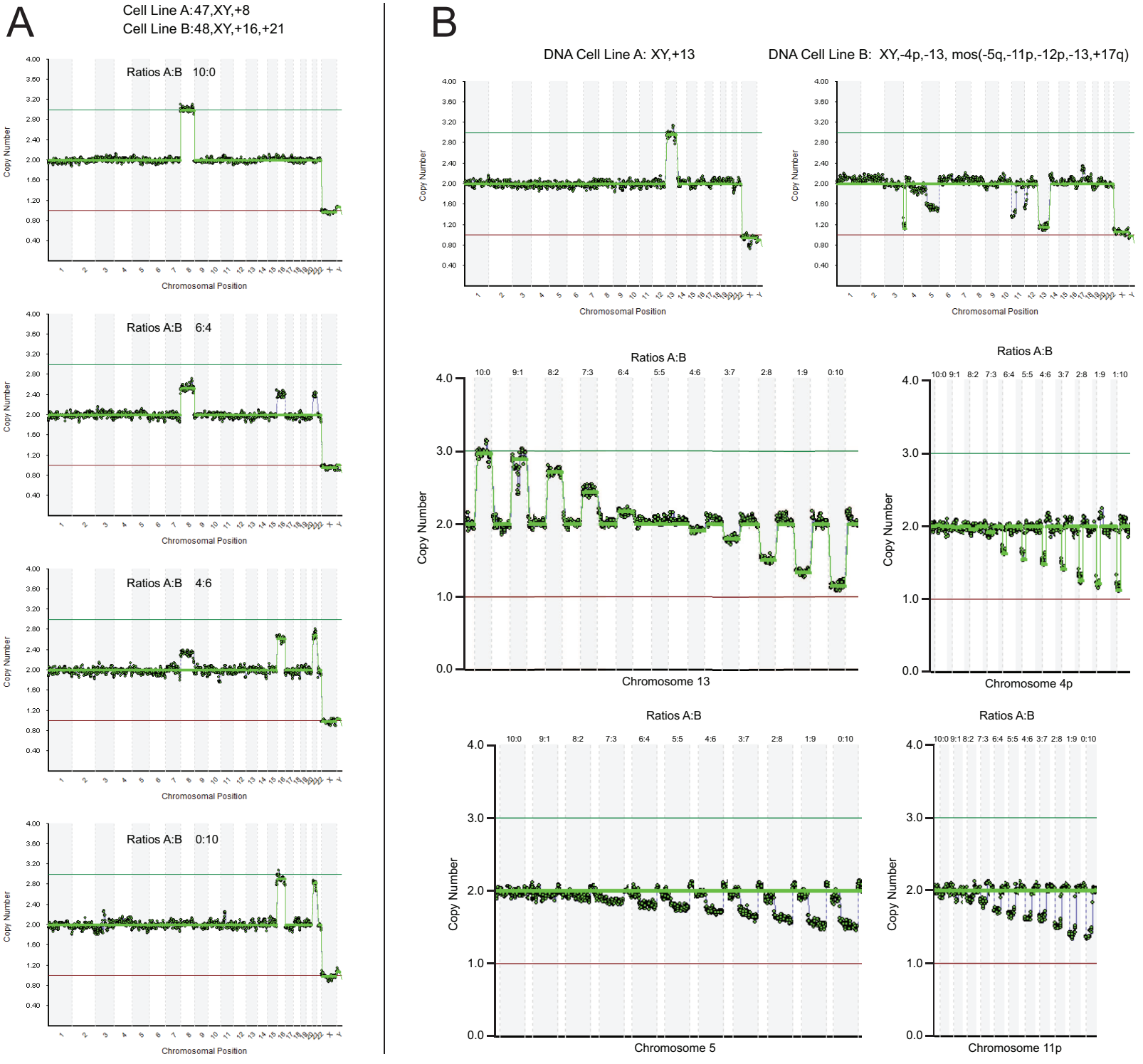

FIG1

Figure2
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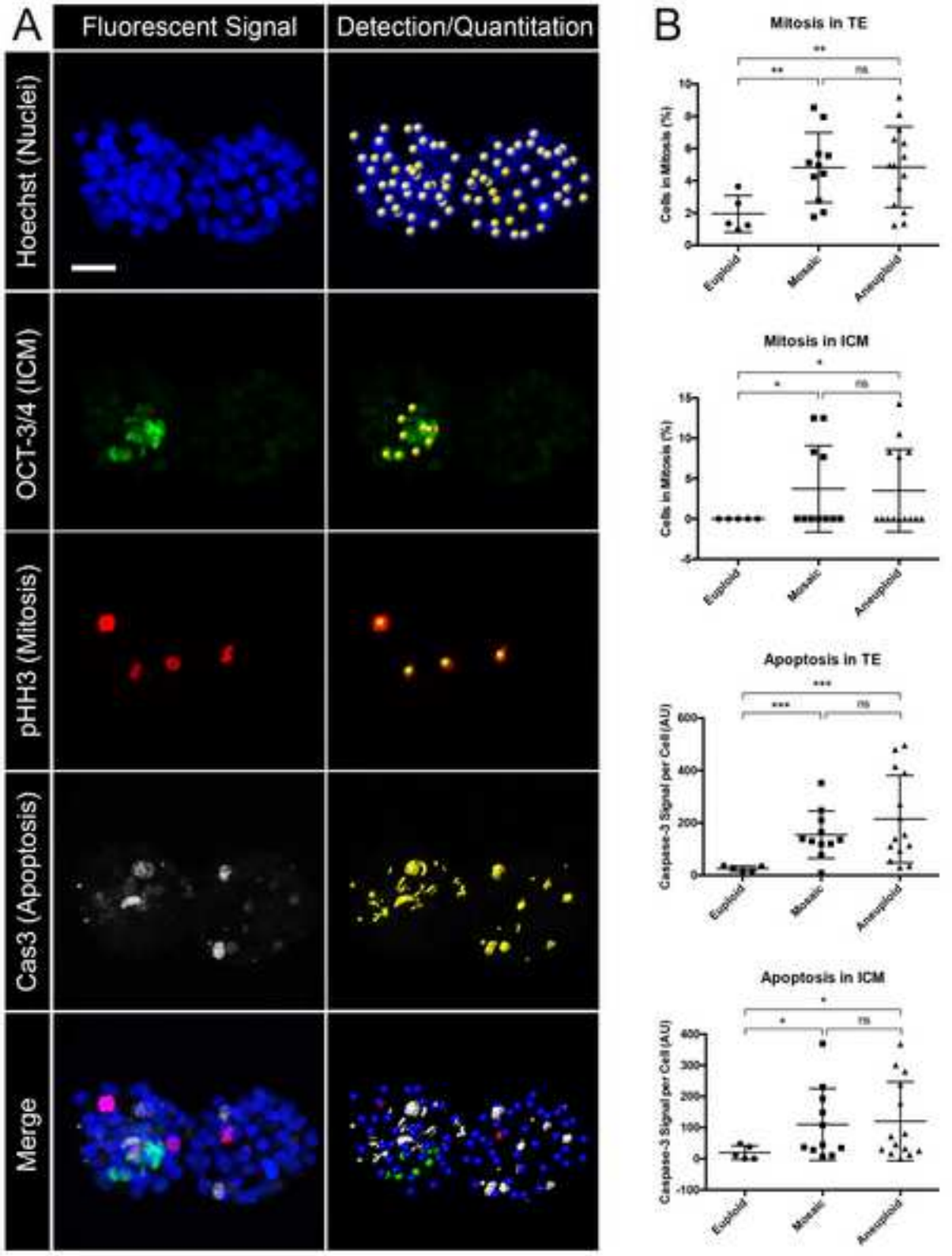

FIG2

Table1

TABLE 1

Embryo Number	Classification	Reported Summary	Beta-hCG +	Sac (Implantation)	Fetal Heart beat	Birth	Mosaic Level[1]	Embryo Evaluation	Oocyte Age	Age Embryo Recipient	Number of Previous FETs	Post implantation Testing
Single Mosaic FETs												
1	Single Seg	mos(+9p)	Y	N	N	N	50%	5BB	30	30	0	
2	Single Seg	mos(-9p)	N	N	N	N	40%	5BB	37	37	3	
3	Single Seg	mos(-14q31.1q32.33)	N	Y	N	N	35%	6BB	37	37	1	
4	Single Seg	mos(-9q21.33q34.3)	Y	Y	Y	Y	50%	5BC	43	43	1	
5	Single Seg	mos(+5q12.3q35.3)	N	N	N	N	20%	5BC	40	40	0	
6	Single Seg	mos(+9q21.13q34.3)	Y	Y	Y	Y	30%	5BB	39	39	1	
7	Single Seg	mos(-4q22.2q35.2)	Y	N	N	N	20%	5BB	36	36	0	
8	Single Seg	mos(+3p24.1p21.1)	N	N	N	N	25%	5CC	41	41	1	
9	Single Seg	mos(+21p11.1q22.11)	Y	Y	Y	ongoing	30%	5BB	37	34	0	NIPT:Normal
10	Single Seg	mos(+19p)	N	N	N	N	30%	5BB	42	42	1	
11	Single Seg	mos(+Xq22.3q28)	Y	Y	Y	ongoing[a]	50%	5BB	42	42	0	NIPT:Normal
12	Single Seg	mos(-Xq)	N	N	N	N	25%	5BC	39	42	0	
13	Single Seg	mos(+1p36.33p34.3)	Y	Y	Y	ongoing	70%	5BC	33	33	0	
14	Single Seg	mos(-7q)	Y	N	N	N	35%	5BB	39	39	2	
15	Single Seg	mos(-Xq21.1q28)	Y	Y	Y	ongoing	30%	5BB	40	33	0	
16	Single Seg	mos(-5q14.3q35.3)	N	N	N	N	20%	5BB	39	39	1	
17	Multiple Seg	mos(-2p25.3p24.1,-21q22.1q22.3)	Y	Y	Y	Y	40%	5AB	33	33	2	Amnio:Normal
18	Multiple Seg	mos(+4q22.1q32.3,-4q32.3q35.2)	N	N	N	N	60%	5BC	39	39	0	
19	Multiple Seg	mos(-1p36.11p32.3,-5p13.3q14.3)	N	N	N	N	40%	6CC	40	40	0	
20	Multiple Seg	mos(-4p16.3q22.1,+4q22.1q35.2)	N	N	N	N	50%	6CB	39	39	1	
21	Multiple Seg	mos(-2p25.3p23.1,-8p)	N	N	N	N	30%	5BC	36	42	2	
22	Multiple Seg	mos(-1q,-9q21.2q31.2)	N	N	N	N	50%	5BB	34	34	1	
23	Multiple Seg	mos(+4q32.3qter,-10q22.2qter)	Y	Y	Y	ongoing	40%	5AB	24	36	6	NIPT:Normal
24	1 or 2 whole	mos(+14)	N	N	N	N	50%	5CC	31	31	1	
25	1 or 2 whole	mos(-15)	N	N	N	N	30%	5BB	36	36	0	
26	1 or 2 whole	mos(-20)	Y	N	N	N	20%	6BC	31	52	3	
27	1 or 2 whole	mos(+6)	N	N	N	N	20%	5BC	38	38	1	
28	1 or 2 whole	mos(+3)	Y	Y	Y	Y	60%	5BC	33	26	3	NIPT:Normal; Amnio:Normal
29	1 or 2 whole	mos(-13)	N	N	N	N	50%	4BB	37	37	0	
30	1 or 2 whole	mos(+3)	N	N	N	N	25%	4CC	39	39	1	
31	1 or 2 whole	mos(-3)	N	N	N	N	35%	4BB	34	34	0	
32	1 or 2 whole	mos(+17)	N	N	N	N	25%	5BC	42	42	0	
33	1 or 2 whole	mos(-X)	Y	N	N	N	25%	5BB	33	33	3	
34	1 or 2 whole	mos(-11)	Y	Y	N	N	40%	5BB	38	38	3	
35	1 or 2 whole	mos(+9)	N	N	N	N	40%	4BC	44	44	0	
36	1 or 2 whole	mos(-6)	N	N	N	N	50%	5BC	37	37	0	
37	1 or 2 whole	mos(+14)	N	N	N	N	20%	5BB	43	43	1	
38	1 or 2 whole	mos(-18)	Y	Y	Y	ongoing	20%	6BA	43	25	0	NIPT:Normal
39	1 or 2 whole	mos(-7,+22)	N	N	N	N	45%	6AA	35	35	3	
40	1 or 2 whole	mos(+2,-15)	Y	N	N	N	25%	5BC	43	43	0	
41	1 or 2 whole	mos(-2,-8)	Y	Y	Y	ongoing	30%	5BB	34	34	2	Amnio: microdeletion[b]
42	1 or 2 whole	mos(+1,-20)	Y	Y	Y	ongoing	60%	5BB	39	39	2	NIPT:Normal
43	1 or 2 whole	mos(+22)	Y	N	N	N	30%	5BB	41	41	2	
44	Complex	mos(+1,+20,-22)	Y	Y	N	N	30%	5BB	34	34	1	
45	Complex	mos(-9,-11,-18;+19)	Y	Y	Y	Y	40%	6BC	34	34	0	NIPT:Normal
46	Complex	mos(-3,+4,+16)	Y	N	N	N	30%	5BC	35	35	0	
47	Complex	mos(-3,-6,-8,-15,-18)	N	N	N	N	40%	5BC	38	38	1	
48	Complex	mos(+3,+5p15q14.3,+19)	Y	Y	Y	Y	35%	5BC	42	42	0	
49	Complex	mos(-1p,+13,+14,+15,+20,+22)	Y	Y	N	N	50%	5BC	41	41	0	
50	Complex	mos(+1,-7,-16,+17,+21q22.12q22.3,+22)	Y	Y	Y	ongoing	30%	3BB	26	45	0	
Double FET (1 Mosaic transferred together with 1 Euploid Embryo)												
51	Single Seg	mos(+11p)	Y	N	N	N	30%	5CC	41	41	0	
52	Single Seg	mos(+12q14.1q24.31)	Y	Y	Y	Y	30%	5BC	37	37	0	
53	Single Seg	mos(-5q)	N	N	N	N	45%	5BB	34	34	1	
54	Single Seg	mos(-1p36.33p31.1)	Y	Y	Y	Y	25%	5AB	34	34	2	Amnio:Normal
55	Single Seg	mos(+1p36.31p32.2)	N	N	N	N	40%	5BB	35	35	1	
56	Single Seg	mos(-10q23.1q26.3)	N	N	N	N	30%	5BB	41	41	1	
57	Single Seg	mos(+6q22.31qter)	(Y)	N	N	N	40%	5BC	37	37	1	
58	Single Seg	mos(-Xq)	(Y)	(Y)	(Y)	(ongoing)	25%	3BB	42	42	1	
59	Multiple Seg	mos(-1q21.3qter,-21p11.1q21.2)	N	N	N	N	30%	5BB	31	31	1	
60	1 or 2 whole	mos(-17)	Y	Y	Y	Y	30%	6BB	39	39	1	Amnio: translocation[c]
61	1 or 2 whole	mos(-14)	N	N	N	N	20%	5BC	29	29	0	
62	1 or 2 whole	mos(+17)	N	N	N	N	25%	5CC	39	39	1	
63	1 or 2 whole	mos(-16)	Y	Y	Y	ongoing	40%	5BB	24	46	0	
64	1 or 2 whole	mos(-21)	(Y)	N	N	N	25%	5BB	38	38	3	
65	1 or 2 whole	mos(+15q14q22.31,+21)	N	N	N	N	35%	5CC	35	35	2	
66	1 or 2 whole	mos(-17p13.2q25.3,+1p31.3p21.3)	N	N	N	N	45%	6CB	40	40	2	
67	1 or 2 whole	mos(-10,+5)	Y	Y	Y	Y	20%	5BB	33	33	1	Amnio:Normal
68	Complex	mos(-14,-18q,+18p)	N	N	N	N	65%	4CC	40	40	0	
Double FET (2 Mosaic)												
69	Complex	mos(-1,+13,-20)	Y	Y	Y	Y	20%	5BC	34	34	1	Amnio:Normal
70	1 or 2 whole	mos(-2)	N	N	N	N	25%	5CC	34	34	1	
71	Multiple Seg	mos(-3p;+21p11.1q21.2)	(Y)*	(Y)*	N	N	40%	5BC	26	32	2	
72	1 or 2 whole	mos(-11)	(Y)*	(Y)*	N	N	25%	5BC	26	26	2	
73	1 or 2 whole	mos(+3p14.3p12.1,+7)	(Y)*	(Y)*	(Y)*	(Y)*	35%	5BB	33	33	1	(Amnio:Normal)*
74	1 or 2 whole	mos(+22)	(Y)*	(Y)*	(Y)*	(Y)*	30%	5BB	33	33	1	(Amnio:Normal)*
75	Single Seg	mos(+16p)	Y	Y	Y	Y	30%	5BB	32	32	1	Amnio: microdeletion[c]
76	1 or 2 whole	mos(-19)	Y	Y	Y	Y	30%	5BC	32	32	1	
77	Multiple Seg	mos(+1p,-1q)	Y	Y	Y	Y[e]	30%	5AB	34	35	2	
78	Single Seg	mos(+11p11.2q14.1)	Y	Y	Y	Y[e]	30%	6BA	34	35	2	
79	Complex	mos(+12,+14,+16,+18,+19,-X)	(Y)*	(Y)*	(Y)*	(Y)*	50%	3BC	36	36	5	(Amnio:Normal)*
80	1 or 2 whole	mos(+16)	(Y)*	(Y)*	(Y)*	(Y)*	40%	5CB	36	36	5	(Amnio:Normal)*
81	Single Seg	mos(-2q35q37.3)	(Y)*	(Y)*	N	N	50%	5BC	36	42	1	
82	1 or 2 whole	mos(-4)	(Y)*	(Y)*	N	N	25%	5BB	36	42	1	
83	Complex	mos(+3,+6,-14,-15,+20)	N	N	N	N	60%	5CC	27	39	0	
84	Complex	mos(+8,+12,-21)	N	N	N	N	50%	4BC	27	39	0	
85	Single Seg	mos(+10q21.3q26.3)	Y	Y	Y	Y	45%	5BB	36	36	1	Amnio:Normal
86	Single Seg	mos(-1p36.33p32.3)	Y	Y	Y	Y	60%	5BB	36	36	1	Amnio:Normal
87	Single Seg	mos(-2p25.3p24.3)	(Y)*	(Y)*	N	N	25%	4BB	34	34	0	
88	1 or 2 whole	mos(+2)	(Y)*	(Y)*	N	N	25%	5CB	34	34	0	
89	Complex	mos(+11p11.2q12.2,+20,+22)	(Y)*	N	N	N	25%	5CC	38	28	7	
90	1 or 2 whole	mos(+19)	(Y)*	N	N	N	30%	5BB	38	28	7	
91	Single Seg	mos(-5p)	N	N	N	N	40%	3CC	39	39	0	
92	Single Seg	mos(+14q32.12q32.33)	N	N	N	N	35%	5CB	39	39	0	
93	Multiple Seg	mos(+3p14.2p14.1,+16p13.3p12.1,+18q11.2q24.3	(Y)*	(Y)*	(Y)*	(onging)*	50%	5CC	42	42	1	
94	1 or 2 whole	mos(-3,-X)	(Y)*	(Y)*	(Y)*	(onging)*	20%	5BC	42	42	1	
Triple FET (3 Mosaic)												
95	Single Seg	mos(+13q13.3q33.3)	(Y)[#]	N	N	N	20%	5CC	40	40	1	
96	1 or 2 whole	mos(+5,+20)	(Y)[#]	N	N	N	40%	5CC	40	40	1	
97	1 or 2 whole	mos(+19)	(Y)[#]	N	N	N	50%	5BC	40	40	1	
98	1 or 2 whole	mos(-7,-17)	(Y)[#]	(Y)[#]	N	N	50%	5BB	40	40	3	
99	1 or 2 whole	mos(-4)	(Y)[#]	(Y)[#]	N	N	30%	5BB	40	40	3	
100	1 or 2 whole	mos(+21)	(Y)[#]	(Y)[#]	N	N	40%	5BB	40	40	3	

Table2

TABLE 2

Type	Embryos Transferred	Beta-hCG +	Sac [Implantation]	Fetal Heartbeat	Beta-hCG + (%)	Sac (%) [Implantation]	Fetal Heartbeat (%)	P Value Sac [Implantation]	P Value Fetal Heartbeat	Average Age	Average Mosaicism
Euploid All	478	296	237	225	61.9%	49.6%	47.1%			37.4	N/A
Euploid No Selection	109	68	49	46	62.4%	45.0%	42.2%	0.3974 (ns) [a]	0.3949 (ns) [a]	36.9	N/A
Mosaic All	100	49	37	30	49.0%	38.0%	30.0%	0.0273 (*) [a]	0.0019 (**) [a]	36.4	36%
Mos. Single Segmental	33	19	15	13	57.6%	45.5%	39.4%	0.7203 (ns) [a]	0.4717 (ns) [a]	37.6	35%
Mos. Multiple Segmental	11	4	4	3	36.4%	36.4%	27.3%	0.5446 (ns) [a]	0.2331 (ns) [a]	34.4	42%
Mos. 1 or 2 Whole Chr.	43	18	12	10	41.9%	27.9%	23.3%	0.0067 (**) [a]	0.0035 (**) [a]	36.6	33%
Mos. Complex (>2 Chr)	13	8	6	4	61.5%	46.2%	30.8%	1.0000 (ns) [a]	0.2748 (ns) [a]	34.7	40%

Level Range of Mosaicism	Embryos Transferred	Beta-hCG +	Sac [Implantation]	Fetal Heartbeat	Beta-hCG + (%)	Sac (%) [Implantation]	Fetal Heartbeat (%)	P Value Sac [Implantation]	P Value Fetal Heartbeat	Average Age	
20% – 50%	78	39	28	23	50.0%	35.9%	29.5%			36.4	-
50% – 80%	22	10	9	7	45.5%	40.9%	31.8%	0.8031 (ns) [b]	0.7992 (ns) [b]	36.5	-
20% – 40%	58	32	21	16	55.2%	36.2%	27.5%			36.7	-
40% – 80%	42	17	16	14	40.5%	38.1%	33.3%	1.0000 (ns) [b]	0.6590 (ns) [b]	36.0	-

Age of Oocyte (years)	Embryos Transferred	Beta-hCG +	Sac [Implantation]	Fetal Heartbeat	Beta-hCG + (%)	Sac (%) [Implantation]	Fetal Heartbeat (%)	P Value Sac [Implantation]	P Value Fetal Heartbeat		Average Mosaicism
≤34 Euploid	141	95	72	69	67.4%	51.1%	48.9%			-	N/A
>34 Euploid	337	201	165	156	59.6%	49.0%	46.2%	0.6893 (ns) [b]	0.6164 (ns) [b]	-	N/A
≤34 Mosaic All	34	21	19	16	61.8%	55.9%	47.1%			-	35%
>34 Mosaic All	66	28	18	14	42.4%	27.3%	21.2%	0.0082 (**) [b]	0.0111 (*) [b]	-	36%

Mosaic Abnormality	Embryos Transferred	Beta-hCG +	Sac [Implantation]	Fetal Heartbeat	Beta-hCG + (%)	Sac (%) [Implantation]	Fetal Heartbeat (%)	P Value Sac [Implantation]	P Value Fetal Heartbeat	Average Age	Average Mosaicism
Gain	38	18	13	12	47.4%	34.2%	31.6%			37.8	35%
Loss	42	20	16	11	47.6%	38.1%	26.2%	0.8172 (ns) [b]	0.6296 (ns) [b]	36.2	33%

[c]Blastocysts with PGT-A Classification: Mosaic Only

	Blastocyst 1	Blastocyst 2	Blastocyst 3	Blastocyst 4	Blastocyst 5
Clinical TE Biopsy	XY, mos(-10 [*50%*])	XY, mos(+15 [*50%*])	XX, mos(-3 [*65%*])	XY, mos(+3 [*65%*], +9 [*40%*], +11 [*45%*])	XX, mos(-12 [*40%*], +18 [*45%*], +21[*50%*])
ICM Biopsy	XY, euploid	XY, mos(-15 [*50%*])	XX, mos(+3 [*40%*])	XY, euploid	XX, euploid
Second TE Biopsy	XY, mos(+10 [*25%*])	XY, mos(-15 [*50%*])	XX, mos(+3 [*75%*])	XY, euploid	XX, euploid

[c]Blastocysts with PGT-A Classification: Uniform Aneuploid and Mosaic

	Blastocyst 6	Blastocyst 7	Blastocyst 8
Clinical TE Biopsy	XX,+22, mos(+1q21.2-q44 [*70%*])	XX, -22, mos(-10 [*80%*])	XX,+14, -21, mos(-16q [*30%*])
ICM Biopsy	XX, +22	XX, -22, mos(+1 [*30%*], -X [*25%*])	XX,+14, -21, mos(-16q [*65%*])
Second TE Biopsy	n/a	XX, -22, mos(+19 [*35%*])	XX,+14, -21, mos(-16q [*50%*])

Table Suppl. 1

Transfer #	Mosaic Embryo Grade	Euploid Embryo Grade	Beta-hCG	Mosaic Sac	Euploid Sac	Mosaic Heartbeat	Euploid Heartbeat
1	5BC	5AB	+	Y	Y	Y	Y
2	5AB	5BB	+	Y	Y	Y	Y
3	5BB	5BB	+	Y	Y	Y	Y
4	6BB	4CC	+	Y	N	Y	N
5	5BB	5CC	+	Y	N	Y	N
6	5CC	5CB	+	N	N	N	N
7	3BB	6CB	+	(Y)	(Y)	(Y)	(Y)
8	5BC	5BC	+	N	N	N	N
9	5BB	5BB	+	(Y)	(Y)	N	N
10	5CC	5CC	-	N	N	N	N
11	6CB	5BB	-	N	N	N	N
12	5BB	5CC	-	N	N	N	N
13	5BB	5CC	-	N	N	N	N
14	4CC	5CC	-	N	N	N	N
15	5CC	5BB	-	N	N	N	N
16	5BB	5BB	-	N	N	N	N
17	5BC	4CC	-	N	N	N	N
18	5BB	5BC	-	N	N	N	N

Table Suppl. 2

Age of Oocyte (years)	Embryos Transferred	Beta-hCG +	Sac [Implantation]	Fetal Heartbeat	Beta-hCG + (%)	Sac (%) [Implantation]	Fetal Heartbeat (%)	P Value Sac [Implantation]	P Value Fetal Heartbeat	Average Mosaicism
≤34 Euploid	141	95	72	69	67.4%	51.1%	48.9%			N/A
>34 Euploid	337	201	165	156	59.6%	49.0%	46.2%	0.6893 (ns) [b]	0.6164 (ns) [b]	N/A
≤34 Mosaic All	34	21	19	16	61.8%	55.9%	47.1%			35%
>34 Mosaic All	66	28	18	14	42.4%	27.3%	21.2%	0.0082 (**) [b]	0.0111 (*) [b]	36%
≤34 Mos. Single Segm.	7	6	5	4	85.7%	71.4%	57.1%			39%
>34 Mos. Single Segm.	26	13	10	9	50.0%	38.5%	34.6%	0.2028 (ns) [b]	0.3926 (ns) [b]	34%
≤34 Mos. Multi Segm.	6	3	3	3	50.0%	50.0%	50.0%			38%
>34 Mos. Multi Segm.	5	1	1	1	20.0%	20.0%	20.0%	0.5455 (ns) [b]	0.5455 (ns) [b]	46%
≤34 Mos. 1 or 2 Whole Chr.	15	9	7	6	60.0%	46.7%	40.0%			31%
>34 Mos. 1 or 2 Whole Chr.	28	9	5	4	32.1%	17.9%	13.0%	0.0739 (ns) [b]	0.0726 (ns) [b]	34%
≤34 Mos. Complex	6	4	4	3	66.7%	66.7%	50.0%			38%
>34 Mos. Complex	7	4	2	1	57.1%	28.5%	14.3%	0.2861 (ns) [b]	0.2657 (ns) [b]	42%

Figure Suppl. 1