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ORIGINAL ARTICLE *Embryology*

Euploidy rates in donor egg cycles significantly differ between fertility centers

S. Munné[1,*], M. Alikani[2], L. Ribustello[1], P. Colls[1], Pedro A. Martínez-Ortiz[3], Referring Physician Group[†], and D.H. McCulloh[4]

[1]Reprogenetics, 3 Regent Street Suite 301 Livingston, NJ 07039, USA [2]Reproductive Science Center of New Jersey, 234 Industrial Way, Suite A104, Eatontown, NJ 07724, USA [3]University of Alicante, Carretera San Vicente s/n, San Vicent del Raspeig, Alicante 03690, Spain [4]NYU Langone Medical Center, NYU Fertility Center, 660 First Avenue, fifth floor, New York, NY 10016, USA

*Correspondence address: Reprogenetics, 3 Regent Street, Suite 301, Livingston NJ 07039, USA. Tel: +973-436-5010; Fax: +973-992-1423; E-mail: santi@reprogenetics.com

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STUDY QUESTION: Do external factors affect euploidy in egg donor cycles?

SUMMARY ANSWER: The study demonstrates that during human assisted reproduction, embryonic chromosome abnormalities may be partly iatrogenic.

WHAT IS KNOWN ALREADY: Chromosome abnormalities have been linked in the past to culture conditions such as temperature and Ph variations, as well as hormonal stimulation. Those reports were performed with older screening techniques (FISH), or ART methods no longer in use, and the subjects studied were not a homogeneous group.

STUDY DESIGN, SIZE, DURATION: A total of 1645 donor oocyte cycles and 13 282 blastocyst biopsies from 42 fertility clinics were included in this retrospective cohort study. Samples from donor cycles with PGS attempted between September 2011 and July 2015 were included.

PARTICIPANTS/MATERIALS, SETTING, METHODS: PGS cycles from multiple fertility clinics referred to Reprogenetics (Livingston, NJ) that involved only oocyte donation were included in this study. Testing was performed by array comparative genomic hybridization (aCGH). Ploidy data were analyzed using Generalized Linear Mixed Models with logistic regression using a logit link function considering a number of variables that represent fixed and random effects.

MAIN RESULTS AND THE ROLE OF CHANCE: Euploidy rate was associated with the referring center and independent of almost all the parameters examined except donor age and testing technology. Average euploidy rate per center ranged from 39.5 to 82.5%. The mean expected rate of euploidy was 68.4%, but there are variations in this rate associated with the center effect.

LIMITATIONS, REASONS FOR CAUTION: Data set does not include details of the donor selection process, donor race or ethnic origin, ovarian reserve or ovarian responsiveness. Due to the retrospective nature of the study, associations are apparent, however, causality cannot be established. Discrepancies in regard to completeness and homogeneity of data exist due to data collection from over 40 different clinics.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first study to show a strong association between center-specific ART treatment practices and the incidence of chromosome abnormality in human embryos, although the meiotic or mitotic origin of these abnormalities could not be determined using these technologies. Given the widespread applications of ART in both subfertile and fertile populations, our findings should be of interest to the medical community in general as well as the ART community in particular.

STUDY FUNDING/COMPETING INTEREST(S): No external funds were used for this study. S. Munne is a founding principle of Reprogenetics/current employee of Cooper Genomics. M Alikani's spouse is a founding principle of Reprogenetics/current consultant for Cooper Genomics. The remaining authors have no conflicts to declare.

Key words: aneuploidy / donor oocytes / ART / PGS / euploidy / mosaicism

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Introduction

Genetic screening of thousands of human embryos in the past two decades has clearly established that the incidence of numeric chromosome abnormalities is very high following ovarian stimulation and IVF, overall exceeding 50% of biopsied embryos (Munné *et al.*, 1995; Magli *et al.*, 2001; Ata *et al.*, 2012). A majority of aneuploidies are meiotic in origin and known to be maternal age related (Hassold *et al.*, 1980). Other chromosome abnormalities occur post activation, during resumption of meiosis II and subsequent mitoses, leading to complex abnormalities, polyploidy and mosaicism in resulting embryos (Munné *et al.*, 1994; Munné, 2006; Magli *et al.*, 2007). Post-meiotic abnormality rates are constant across age groups (~35% in Day 3 embryos), while meiotic aneuploidy rates increase with advancing maternal age, from 29% in women <35 years of age to 47% in women 40 years and older (Munné *et al.*, 2007).

Experimental evidence and clinical data suggest that, apart from maternal age, a number of treatment-related factors may affect the incidence of chromosomal imbalance in embryos. These factors include culture conditions and gamete manipulation, *in vitro* gamete ageing, high oxygen tension during culture and immaturity or post maturity of oocytes at the time of fertilization (Badenas *et al.*, 1989; McKiernan and Bavister, 1990; Pickering *et al.*, 1990; Santaló *et al.*, 1992; Almeida and Bolton, 1995; Munné *et al.*, 1997). Other studies have found significant differences in chromosome abnormalities depending on medication type (Weghofer *et al.*, 2008, 2009) or dosage (Baart *et al.*, 2007; Rubio *et al.*, 2010) for ovarian stimulation.

One study using fluorescent *in situ* hybridization (FISH) to assess whole cleavage stage human embryos suggested that the incidence of mosaicism differed among different fertility centers (Munné *et al.*, 1997). Munné *et al.* (2006) also examined chromosome abnormality rates in egg donors and found significant differences between cycles in the same donor as well as differences between donors. However, all these studies had multiple limitations, including the FISH technique (which did not analyze all chromosomes), single blastomere biopsy in cleavage stage embryos and small or heterogeneous population of patients examined.

In the nearly two decades that have passed, improvements have been made in both diagnostic and embryo culture technologies: With the introduction of array comparative genomic hybridization (aCGH) (Gutiérrez-Mateo *et al.*, 2011; Ata *et al.*, 2012), single nucleotide polymorphism (SNP) arrays (Treff *et al.*, 2010; Rabinowitz *et al.*, 2012), quantitative PCR (Treff *et al.*, 2012) and next generation sequencing (NGS) (Fiorentino *et al.*, 2014; Wells *et al.*, 2014), combined with trophectoderm biopsy (McArthur *et al.*, 2005; Scott *et al.*, 2013), the question of whether some abnormalities detected in early human embryos are treatment-related deserves to be reinvestigated.

Accordingly, we initiated a retrospective analysis of trophectoderm biopsy results for oocyte donation cycles attempted in multiple fertility clinics, asking whether we could identify variation in the level of euploidy among the clinics. Oocyte donors are young, presumably fertile women in whom oocyte chromosome abnormalities are expected to be low (<40%; Ata *et al.*, 2012). Thus, the study population is relatively homogeneous. All genetic analyses of biopsies were conducted at a single reference laboratory, eliminating technical variation in testing as a confounder of euploidy rates. We found that the variable 'referring center' had a significant influence on euploidy rate, in addition to the expected effect of maternal age and changes in technology through time.

Material and Methods

Patient selection

PGS cycles from multiple fertility clinics referred to Reprogenetics (Livingston, NJ) between September 2011 and July 2015 and that involved oocyte donation were included in this study. Oocyte donors were selected by each fertility center; the reasons for offering PGS for egg donation cycles were not specified.

Signed informed consent for the PGS procedure was obtained from oocyte recipients by each center and sent to Reprogenetics for all patients. As part of the standard PGS referral procedure, age of donors and recipients and biopsy data were shared with Reprogenetics. Patient data were de-identified by the Principal Investigator after the PGS procedure and the PGS results were compiled for analysis. This investigation was approved by Aspire IRB as a retrospective study of archived clinical data with de-identification (Protocol number PGSP-2015).

Analysis of samples

Blastocyst biopsy was performed by each clinic at the blastocyst stage, either on Day 5 or Day 6 of development. All centers used a laser to perform the biopsy. Biopsied samples were sent to Reprogenetics for analysis by array CGH. Biopsy methodologies were most likely not identical; the impact of the procedure itself on the diagnosis (Capalbo *et al.*, 2015) was not considered here but will be assessed in future studies. However, embryos with a diagnosis of degraded DNA were excluded from the analysis based on the possibility that poor technique could lead to this outcome. Array CGH has been extensively validated in single-cell biopsies as well as multi-cell blastocyst biopsies and in both instances, the misdiagnosis rate was below 2% (Colls *et al.*, 2012; Gutiérrez-Mateo *et al.*, 2011; Capalbo *et al.*, 2013; Kung *et al.*, 2015).

It should be noted that there are two other potential sources of uncertainty in this data set: first, a limited number of vitrified/warmed oocyte donation cycles were included in the database but these were not distinguishable. However, aneuploidy rates have not been shown to increase following oocyte vitrification (Forman *et al.*, 2012; Goldman *et al.*, 2015). Second, oocytes from one donor cycle may have been used for multiple recipients but because this was not specified in the database, in the analyses, each cycle was treated as an independent cycle.

Samples were classified as euploid if they had the same chromosome content as the control DNA, that is 46,XX or 46,XY, aneuploid if they had an extra or missing chromosome, or segmental abnormal if they had a missing or extra piece of chromosome above 6 MB in size. aCGH can occasionally identify blastocyst biopsies as mosaic (4% according to Greco *et al.*, 2015) and in those instances the criteria at the time were to classify these embryos as abnormal. In this study, all chromosome abnormalities (aneuploidy, mosaicism and segmental abnormalities) were grouped together for purposes of analysis.

Array CGH was performed as described previously (Colls *et al.*, 2012; Gutiérrez-Mateo *et al.*, 2011) with some minor modifications. In September 2011, a-CGH was performed using the 24SureV3 (Illumina, San Diego, CA) single channel method, which uses male and female reference DNAs, thus each sample was compared to two male and two female references. The analysis software was BlueFuse (Illumina, San Diego, CA) versions 2.4 (September 2011), 2.6 (December 2011), 3.0 (August 2012), 3.2 (August 2013) and 4.1 (August 2014). From version 2.4 to 2.6, there was no impact on euploidy detection capabilities. The better algorithms included in the later versions (3.0 onwards) of BlueFuse allowed the

detection of partial gains or losses of DNA of a size of 6 MB or larger, which were diagnosed as sub-chromosomal abnormalities. The change in technology was considered as a variable, referred to as 'testing technology' in the current analysis.

Donor and patient demographics were obtained from each clinic either through a questionnaire, the testing requisition form submitted by each center or through the PGS portal of the eIVF database (PracticeHwy, Dallas, TX). Testing results and all other relevant information were stored in a Reprogenetics database, and this database was used for data analysis.

Statistical analysis

The rate of euploidy per cycle was determined by dividing the number of euploid embryos by the sum of euploid and chromosomally abnormal embryos. Embryos with no diagnosis and/or degraded DNA were excluded. The average rate of euploidy per center was determined as the arithmetic average of euploidy rate for all donor cycles at the center.

Cycle-specific variables with fixed and random effects were considered in the analysis. Those considered to be included: testing technology (x_1), age of the donor (x_2), number of embryos with a ploidy diagnosis (x_3), day of biopsy (Day 5 or Day 6) (x_4), diagnosis of male factor (x_5), use of donor sperm (x_6), age of the recipient (x_7) and age of the recipient's male partner (x_8) (female partner age was used to estimate the male partner's age). The referring center was considered a variable with random effect (ε_c). The euploidy state(E) is defined as the dichotomous variable that indicates whether an embryo is euploid ($E = 1$) or not ($E = 0$). We analyzed the euploidy state of the sample by using a generalized linear mixed model (GLMM) with a logistic link function:

$$logit\,(P\,(E = 1)) = \alpha + \sum_{i=1}^{8} \beta_i \cdot x_i + \varepsilon_c + \varepsilon,$$

where α is the interception, β_i the coefficient for each fixed variable x_i and ε is the residual random variable. This GLMM will give insight about which effects (fixed or random) have significant or non-significant influence on euploidy rate.

All fitting was performed to detect parameters that were associated with the incidence of euploidy for each donor cycle. The variables that remain in the model were established by using a backward elimination procedure, removing those the removal of which does not reduce the deviance (imbalance) of the model fit, and which prove to be non-significant.

The Wilcoxon signed-rank test was used to determine differences in euploidy rates among the centers.

Results

Cycles with donors ≥36 years ($n = 19$), unreported donor age or missing biopsy date were excluded from the analysis. One cycle with only undiagnosed blastocysts was not included in the analysis.

The data set thus included a total of 42 centers with 126 physicians referring 1665 PGS cycles for 1549 recipient between September 2011 and July 2015. Average donor age was 25.5 ± 3.03 years (range: 19–35 years).

A total of 13 595 blastocysts were analyzed, of which 313 (2.3%) had no diagnosis, 177 had amplification failure and 136 had degraded DNA. Of the 13 282 diagnosed blastocysts, 9162 (68.98%) were euploid and the rest were chromosomally abnormal. We chose to examine euploidy rate per donor cycle, which ranged from 0 to 100%. Euploidy rates were not normally distributed and average euploidy rate per donor cycle was 68.5 ± 22.2%.

Identifying associations between euploidy and cycle-specific variables

Multiple GLMM with logistic regression was used to examine the relationship between the incidence of euploidy per donor cycle and the independent variables described above. The analyses showed that only two fixed effect variables, namely, donor age ($P = 0.04$) and testing technology ($P = 0.000007$) along with the random effect variable referring center were significant for euploidy. With respect to the referring center, variance was significantly nonzero ($P = 0.002$) thus leading to the conclusion that the center influences the incidence of euploidy. Inclusion of the fixed effect variables in the model provided minimal improvement of 0.21% compared to the simplest model that only considered the random effect represented by the referring center. None of the other variables examined (number of embryos, diagnosis of male factor, use of donor sperm, age of the recipient or age of the recipient's male partner) was significantly associated with the donor cycle-specific euploidy. On the other hand, the correlation intraclass coefficient showed that 8.34% of the variability in euploidy rate is due to the center. The between center variance can also be summarized using the so called median odds ratio (MOR) (Larsen *et al.*, 2000). The estimation of the MOR for this random variable leads to a value of 1.33, showing that (in the median case) the residual heterogeneity between centers increases by 1.33 times the individual odds ratio of euploidy when randomly picking out two patients in different centers. Although it is a modest MOR value, the variation turns out to be significant as the GLMM analysis shows and it deserves to be taken into account for explaining euploidy rate. Inter-center differences are shown in Fig. 1.

To verify that the observed effect of these factors on euploidy rate was not due to other confounding variables, several GLMM models were generated by eliminating these potential confounders gradually. In these models, it was observed that the coefficients associated with these factors were hardly modified (<10%) after removing other factors (both fixed and random). This suggests that there are in fact no confounding variables in the model and that the random factor associated with the center is not an effect derived from other factors that were considered in this study.

Discussion

In this study, we have demonstrated by using a GLMM analysis that the rate of euploidy in embryos generated from donated oocytes is treatment center-dependent.

Our analysis also showed a significant association between donor age as well as testing technology and the diagnosis of euploidy in embryos. It should be noted, however, that our data set did not include details of the donor selection process, whether donors were directed or anonymous, donor race or ethnic origin, ovarian reserve or ovarian responsiveness. We also did not collect information on donor selection criteria used by each center. Potential impact of these factors on chromosome status of donor oocytes would have to be further investigated.

In agreement with a previous study (Ata *et al.*, 2012), we found that the number of blastocysts biopsied in each cycle was not associated with euploidy rate. Although the number of embryos biopsied may not be related to euploidy rates (Ata *et al.*, 2012), there may be an optimal number of eggs for achieving maximum pregnancy rates (Sunkara *et al.*, 2011).

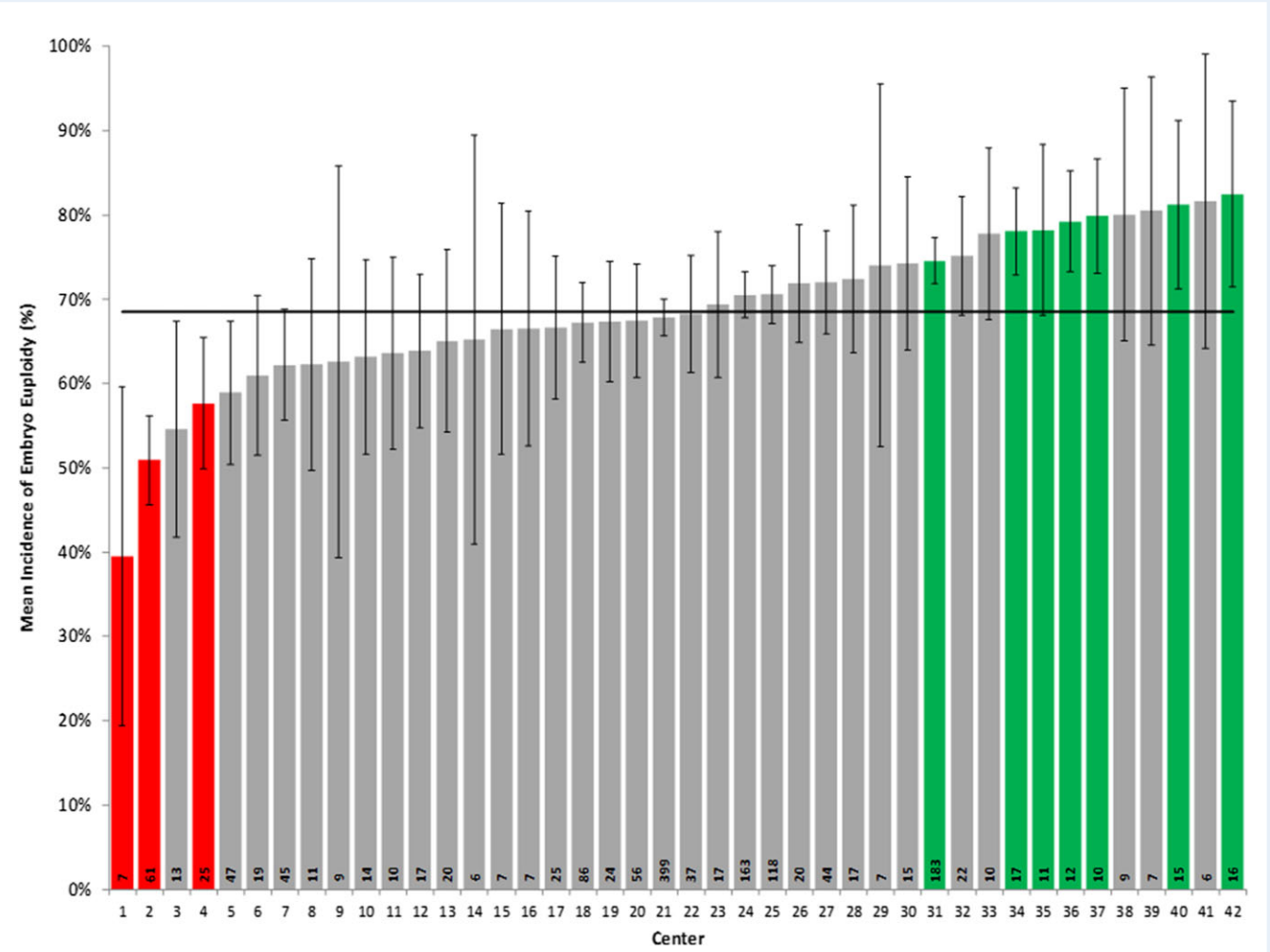

Figure 1 Mean incidence of embryo euploidy (%), calculated for individual centers, weighting each donor cycle equally regardless of the number of embryos per cycle. The mean incidence of donor cycle euploidy for each center is displayed as a shaded bar (error bars represent ±95% confidence limits for the mean value). The overall mean incidence of euploidy for all donors from all centers (68.5%) is displayed as a bold horizontal black line. The number of donor cycles is displayed at the bottom of each center's bar. Centers with an incidence of euploidy that was significantly different from the overall mean incidence of euploidy using Wilcoxon signed-rank test are shaded in red (significantly lower euploidy) or in green (significantly higher euploidy). Centers with an incidence of euploidy that was not significantly different from the overall mean incidence of euploidy are shaded in gray. Center ID number represents the center's ranking from lowest to highest incidence of euploidy.

The observation of Sunkara *et al.* (2011) could mean that hormonal stimulation may affect euploidy independent of embryo number or that it affects uterine receptivity. Corroborating evidence is also provided by the study of Baker *et al.* (2015), showing that live birth rates diminish with increasing dosage of gonadotrophins, nearly independently of maternal age (Baker *et al.*, 2015). Taken together, it may be speculated that differences in donor ovarian stimulation regimens used by different treating centers in this study may be a contributing factor to the observed differences in euploidy rates.

Oocyte donor age was found to affect euploidy rate as expected and shows that the increase in chromosome abnormalities observed with increasing maternal age is already occurring in this young group of oocyte donors.

Testing technology was also expected to produce some differences in chromosome abnormality rates. For example in the earlier part of the study period, segmental abnormalities were not identified but these abnormalities accounted for ~5% of all chromosome abnormalities in later analyses.

Paternal age is known to influence reproductive outcome and thus potentially euploidy (Frattarelli *et al.*, 2008). However, our analyses showed no significant relationship between euploidy rate and male partner age. Likewise, the use of donor sperm did not influence euploidy rate.

Using SNP array analysis of embryos, most embryonic meiotic aneuploidies have been found to be maternal in origin (Treff *et al.*, 2010; Rabinowitz *et al.*, 2012; Konstantinidis *et al.*, 2015), and although male factor infertility can increase chromosome abnormality rates in embryos (Silber *et al.*, 2003; Dubey *et al.*, 2008; Magli *et al.*, 2009; Sánchez-Castro *et al.*, 2009; Rodrigo *et al.*, 2010), male factor was described as a diagnosis in only 9.5% of the cycles in this study. Euploidy rate for these cycles was not significantly different from the incidence expected for the entire donor pool.

Despite differences in the proportion of blastocysts biopsied on Day 5 versus Day 6 at different centers, this proportion was not associated with euploidy rate.

Of the many center-specific factors that could explain the differences among centers, subtleties of case management, such as drug type, dosage and duration for ovarian stimulation, and even follicular aspiration methodologies could influence oocyte chromosome integrity. Physicians may have individual preferences for FSH dosage for donors and the number of eggs they aim to collect. Higher FSH dosages in the presence of normal ovarian response may be associated with poorer outcomes (Baker *et al.*, 2015), which may be oocyte quality related. Physicians may also follow different routines during follicle aspiration, for example which follicles they choose to aspirate and at what negative pressure, whether they tend to flush follicles or not, and differences in time between hCG administration and egg retrieval. Smaller follicles harbor more immature eggs, which have a higher incidence of chromosome abnormalities, and following IVM and IVF, these eggs lead to a higher incidence of multinucleation (De Vincentiis *et al.*, 2013; Alvarez Sedo *et al.*, 2015); multinucleation, in turn, has been associated with a high rate of chromosome abnormality (Kligman *et al.*, 1996; Munné, 2006; Munné *et al.*, 2007). Contaminants and volatile compounds could be also contributing to these differences in aneuploidy rates (Hunt *et al.*, 2003, Susiarjo *et al.*, 2007). On the other hand, some of the differences observed here could be produced by culture conditions and affect post-meiotic abnormalities (Munné *et al.*, 1997; Munné and Alikani, 2011). Indeed, a preliminary study comparing different culture media also detects significant differences in chromosome abnormalities between treatments, although pH values were also different (Hickman *et al.*, 2016), and another study using NGS showed significant differences between centers in aneuploidy and mosaicism rates (Sachdev *et al.*, 2016). Our study, by using aCGH was not able to assess the origin of the abnormalities.

This study has some limitations. First, this is a retrospective observational study. The analyses used in the study can show associations between variables, but they cannot establish causality. Nonetheless, retrospective studies of this type are needed in order to design appropriate prospective, randomized studies that rigorously test the associations of interest. Second, the study uses a large database containing data from >40 clinics. This is an enormous advantage with respect to sample size, but a disadvantage with respect to data completeness and homogeneity. A number of relevant parameters, including stimulation protocols, were not available in this data set and could not be analyzed in this first phase of the study; these will be considered in follow-up studies.

In summary, this is the first study to show a strong association between center-specific ART treatment practices and the incidence of chromosome abnormality in human embryos generated from donated oocytes. Further elucidation of the nature of the different practices that may be contributing to higher or lower euploidy rates should provide opportunities for improving clinical outcomes of ART. Considering the ever-wider applications of ART in both subfertile and fertile populations, our findings should be of interest to the medical community in general as well as the ART community in particular.

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Authors' roles

S.M.: Designed the study, drafted it, approved the submitted versions. M.A.: interpretation of data, drafted it, approved the submitted versions.

P.C.: Performed experiments, critically revised the article, approved the submitted versions. L.R.: Performed experiments, critically revised the article, approved the submitted versions. D.M.: interpretation of data, drafted it, approved the submitted versions. P.A.M-O: designed and performed the complex statistical analysis required by the reviewers, critically revised the article, approved the submitted versions.

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Conflict of interest

S. Munne is a founding principle of Reprogenetics/current employee of Cooper Genomics. M Alikani's spouse is a founding principle of Reprogenetics/current consultant for Cooper Genomics. The remaining authors declare the existence of no conflicts.

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