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# Preliminary assessment of aneuploidy rates between the polar, mid and mural trophectoderm

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**Research Article**

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## Summary

The objective of this study is to compare aneuploidy rates between three distinct areas of the human trophectoderm: mural, polar and a region in between these two locations termed the 'mid' trophectoderm. This is a cohort study on *in vitro* fertilization (IVF) patients undergoing comprehensive chromosome screening at the blastocyst stage at a private IVF clinic. All embryos underwent assisted hatching on day 3 with blastocyst biopsy and comprehensive chromosome screening. Biopsied blastocysts were divided into three groups depending on which area (polar, mid, or mural) of the trophectoderm was protruding from the zona pellucida and biopsied. Aneuploidy rates were significantly higher with cells from the polar region of the trophectoderm (56.2%) compared with cells removed from the mural region of the trophectoderm (30.0%; $P = 0.0243$). A comparison of all three areas combined also showed a decreasing trend, but this did not reach clinical significance, polar (56.2%), mid (47.4%) and mural trophectoderm (30.0%; $P = 0.1859$). The non-concordance demonstrated between polar and mural trophectoderm can be attributed to biological occurrences including chromosomal mosaicism or procedural differences between embryologists.

## Introduction

Aneuploidy refers to the presence or absence of whole chromosomal abnormalities. For a euploid live birth to occur, chromosomes must divide equally in the developing fetus. Any abnormal division during development can have disastrous downstream effects, leading to poor embryo development, failed implantation, obstetric complications, pregnancy loss, stillbirth, neonatal congenital abnormality and infertility. Therefore, preimplantation genetic testing (PGT) has been created to test for aneuploidy prior to implantation, thereby allowing the transfer of euploid embryos. The transfer of euploid embryos has demonstrated a higher pregnancy rate, lower miscarriage rate and higher live birth rate than the transfer of untested embryos (Yang *et al*., 2012; Forman *et al*., 2013; Scott *et al*., 2013). Unfortunately, these studies are limited to good prognosis patients or are not based on 'intent to treat'. More recent research has demonstrated that embryos diagnosed as mosaic, having a mixture of euploid and aneuploid cell lines and even embryos diagnosed as aneuploid can produce live births (Munne *et al*., 2017; Patrizio *et al*., 2019).

The blastocyst represents the first stage of differentiation in preimplantation development. The blastocyst differentiates into the inner cell mass (ICM), which will become the fetus and the trophectoderm, which will become the placenta. The trophectoderm itself is subdivided into two areas based on the location of the ICM: the mural trophectoderm, the area furthest away from the ICM and the polar trophectoderm, the area adjacent to the ICM. Typically, during PGT, cells are removed from the mural trophectoderm to not expose the ICM to the damage caused by the laser (Taylor *et al*., 2014a). However, blastocyst biopsy is not standardized and this lack of standardization can lead to inter- and intra-differences with embryologists in terms of the area biopsied.

It has been suggested that ploidy is consistent throughout the trophectoderm (i.e. that all cells have the same karyotype) (Northrop *et al*., 2010; Capalbo *et al*., 2013). Therefore, cells removed from the mural trophectoderm should mirror the chromosome content of the remaining cells. To test this hypothesis, this study aimed to compare aneuploidy rates between three distinct areas of trophectoderm: mural, polar, and a region in between these two locations termed the 'mid' trophectoderm.

## Materials and methods

This study was deemed exempt by Sterling IRB because it only incorporated routine *in vitro* fertilization (IVF) procedures. Only patients who were undergoing IVF with PGT between

January 2012 and April 2013 at the Reproductive Endocrinology Associates of Charlotte (Charlotte, North Carolina, USA) were included in this study. All biopsy specimens were sent to Genesis Genetics (Detroit, Michigan, USA) where samples underwent next generation sequencing (NGS).

Briefly, all fertilized oocytes were cultured to day 3 and assisted hatching (AH) was performed. Embryos were placed back into incubator and cultured to the blastocyst stage. Embryos whose trophectoderm was hatching out of the zona pellucida (ZP) underwent the biopsy procedure. Biopsied blastocysts were divided into three groups depending on which area (polar, mid, or mural) of the trophectoderm was protruding from the ZP and biopsied.

### *Egg retrieval and embryo culture*

All retrieved oocytes were designated for intracytoplasmic sperm injection (ICSI). Oocytes were retrieved, trimmed of blood and stripped of cumulus cells as described by Taylor and colleagues (Taylor *et al.*, 2008). Oocytes were separated based on maturity and placed into a 60 mm dish (Thermo Scientific, Rochester, New York, USA) with approximately 100 µl drops of continuous culture medium (CSC; Irvine Scientific, Santa Ana, California, USA) supplemented with 10% serum substitute supplement (SSS; Irvine Scientific, Santa Ana, California, USA) and overlaid with oil (Irvine Scientific, Santa Ana, California, USA). After grading, the dish containing the oocytes was placed into an incubator at 37°C, 6% $CO_2$ and 5% $O_2$ in air for 2–3 h. After 2 h, all oocytes presenting with a polar body underwent ICSI as described by Nagy *et al.* (1995), placed back into the same dish and put back into the incubator (1995). Oocytes were placed back into the same dish and put back into the incubator after ICSI.

The next day, 16–18 h post ICSI, oocytes were evaluated for true fertilization. Embryos that exhibited two pronuclei were group cultured in a fresh dish of CSC+10% SSS overlaid with oil and placed back into the incubator. Embryos were not viewed on day 2.

On day 3, the embryos were removed from the incubator, graded and AH was performed on all cleaving embryos with the aid of a laser (Zilos-tk, Hamilton Thorne, Beverly, Massachusetts, USA). Using a pulse of 610 µs, the ZP was breached with two or three shots of the laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA). The ZP was breached where there were no blastomeres that could be directly affected by the laser pulse. After breaching the ZP with the laser, the embryos were left in the same drop and placed back into the incubator.

On the morning of day 5 (112–115 h post insemination) and day 6 (136–139 h post insemination), embryos were removed from the incubator, and blastocysts were graded based on Schoolcraft and colleagues (1999) and those blastocysts that had a good or fair trophectoderm protruding from the ZP, along with good or fair quality ICM, were biopsied. Blastocysts were only viewed once in the morning and at no other times. If the blastocysts were not suitable for biopsy in the morning of day 5, they were re-evaluated on the morning of day 6. Blastocysts were biopsied on day 5 or day 6, depending on the day they met the biopsy criteria. If embryos did not meet the criteria for biopsy on day 6, they were discarded. There was no morphological difference between blastocysts that were biopsied on day 5 or day 6 other than the embryos needed an extra day to reach the proper stage for biopsy.

### *Trophectoderm biopsy*

Blastocysts that presented with a good or fair quality ICM and trophectoderm were placed in a drop of modified human tubal fluid (Irvine Scientific, Santa Ana, California, USA) + 10% SSS (Irvine Scientific, Santa Ana, California, USA). Suction was applied to the blastocysts via a holding pipette (Humagen, Charlottesville, Virginia, USA). A biopsy pipette (Humagen, Charlottesville, Virginia, USA) gently aspirated the trophectoderm into the biopsy needle. A laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA), with a pulse length of 610 µm, was used to 'cut' the trophectoderm from the blastocyst. Without exposing the trophectoderm to unnecessary laser pulses. The piece of trophectoderm was prepped for NGS.

**Table 1.** A comparison of aneuploidy rates between the polar, mid and mural trophectoderm

| | Polar | Mid | Mural | *P*-value |
|---|---|---|---|---|
| Average age (years) | 35.8 ± 4.9 | 34.9 ± 4.4 | 35.2 ± 5.1 | 0.8024[a] |
| No. blastocysts | 48 | 78 | 40 | 0.1859[b] |
| No. aneuploid | 27 (56.2%) | 37 (47.4%) | 12 (30.0%) | |

[a]Kruskal–Wallis test; [b]chi-squared test.

**Table 2.** A comparison of aneuploidy rates between polar and mural trophectoderm

| | Polar | Mural | *P*-value |
|---|---|---|---|
| Average age | 35.8 ± 4.9 | 35.2 ± 5.1 | 0.8417[a] |
| No. blastocysts | 48 | 40 | 0.0243[b] |
| No. aneuploidy | 27 (56.2%) | 12 (30.0%) | |

[a]Kruskal–Wallis test; [b]chi-squared test.

## Results

In total, 166 blastocysts were biopsied, 48 from the polar trophectoderm, 78 from the mid trophectoderm and 40 from the mural trophectoderm. There was no significant difference in maternal age between the three groups, i.e. 35.8 ± 4.9 years, 34.9 ± 4.4 years and 35.2 ± 5.1 years, for the plural, mid and mural trophectoderm, biopsied groups respectively (Table 1; $P = 0.8024$). Aneuploidy rates were 27/48 in the polar trophectoderm group (56.2%), 37/78 in the mid trophectoderm group (47.4%) and 12/40 in the mural trophectoderm group (30.0%; Table 1; $P = 0.1859$). In a direct comparison between mural and polar trophectoderm, aneuploidy rates were significantly higher (Table 2; $P = 0.0243$).

## Discussion

The hypothesis that aneuploidy is evenly distributed throughout the trophectoderm cannot be supported by this study. Aneuploidy rates were significantly higher when cells were taken from the polar region of the trophectoderm (56.2%) compared with cells removed from the mural region of the trophectoderm (30.0%; Table 2). These data also demonstrated a strong trend in decreasing aneuploidy from the polar (56.2%), mid (47.4%) and mural trophectoderm (30.0%; Fig. 1). The non-concordance demonstrated between polar and mural trophectoderm can be attributed to biological occurrences or procedural differences.

Biologically, Hogan and Tilly (1978) dissected mouse ICM from the trophectoderm and left the ICM in culture. Within 5 days, some of the individual ICMs had the appearance of a blastocyst. Moreover, the individual ICMs derived trophoblast giant cells.

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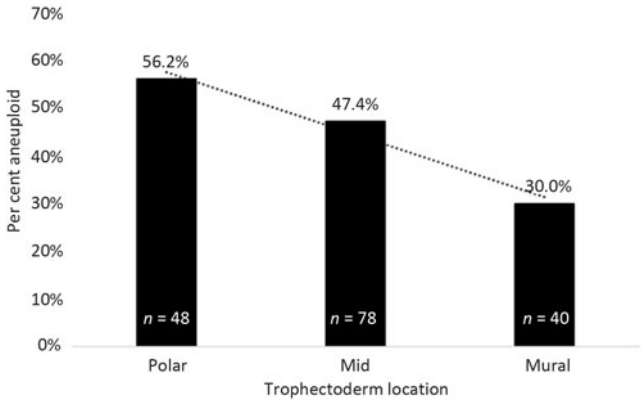


**Figure 1.** Aneuploidy rates between polar, mid and mural trophectoderm.

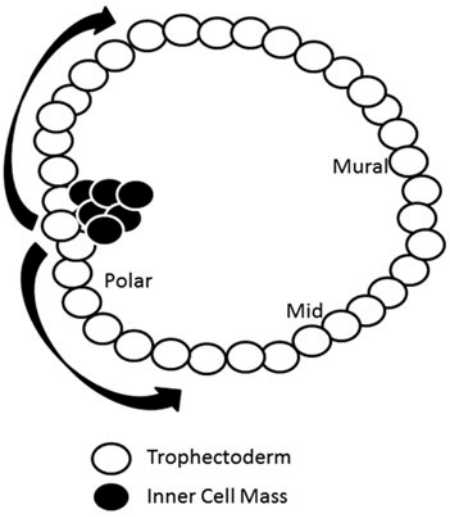


**Figure 2.** Direction of cellular migration from the inner cell mass out into the trophectoderm.

These studies suggested that cells from the ICM feed the trophectoderm. It is unknown if this mechanism is present in human embryos, however if it were this could explain these data. If the ICM were mosaic and contained equal proportions of aneuploid and euploid cells, then aneuploid cells would feed into the trophectoderm at the same rate as euploid cells. Once in the trophectoderm, the euploid cells would proliferate at a faster rate than aneuploid cells (Ruangvutilert *et al.*, 2000). Therefore, the blastocyst could have a higher proportion of aneuploid cells in the polar region compared with the mural trophectoderm, which these data support (Fig. 2). Conversely, this theory would suggest that the blastocyst may be able to allocate aneuploid cells to the trophectoderm, thereby correcting its chromosome state by the elimination of aneuploid cells from the ICM. Research using FISH and array-based techniques have found no evidence of this correction mechanism in place for human blastocysts (Evsikov and Verlinsky, 1998; Johnson *et al.*, 2000; Derhaag *et al.*, 2003; Fragouli *et al.*, 2008; Northrop *et al.*, 2010).

Another biological reason for the discrepancy between regions of the trophectoderm could be the blastocyst preparing for implantation. During implantation, the blastocyst embeds itself with the ICM (polar trophectoderm) against the uterine wall. To invade the uterine wall, the cytotrophoblasts, which are located in the polar region, have been shown to induce aneuploidy (Weier *et al.*, 2005). These data suggest that aneuploidy is higher in the polar region, possibly because the embryo is undergoing chromosomal changes to prepare for implantation. Unfortunately this study did not examine implantation rates between the three different categories, so it is unknown if aneuploidy in the polar region is detrimental. However, transfers of 'aneuploid' or mosaic blastocysts have resulted in euploid live births suggesting that some aneuploidy and mosaicism may not be clinically significant (Scott *et al.*, 2012; Taylor *et al.*, 2014b; Greco *et al.*, 2015; Munne *et al.*, 2017; Patrizio *et al.*, 2019). Both of these biological occurrences suggest that mosaicism is a common phenomenon within the human blastocyst (Taylor *et al.*, 2014b).

The published literature is currently lacking in terms of the effects of the biopsy procedure on the outcomes of PGT cycles. For example, in this study, the embryologist has to biopsy from the mural trophectoderm. Because of its proximity to the ICM, it is possible that some ICM cells were removed with the trophectoderm during the biopsy. Unfortunately, the level of contamination between the ICM and trophectoderm during the biopsy is unknown. However, this may not affect the PGT result, as research has indicated a high concordance between the two regions (Johnson *et al.*, 2000; Capalbo *et al.*, 2013). Interestingly, with the advent of NGS and its increase in the detection of mosaicism, the biopsy procedure has become a variable. If embryologist 'A' biopsies two cells from the blastocyst and both are normal or abnormal, mosaicism will not be detected. However, if embryologist 'B' biopsies 10 cells from the blastocyst and six cells are aneuploid and four cells are euploid, mosaicism will be detected simply due to the increase number of cells biopsied. Research has also suggested that the majority of abnormalities at the blastocyst stage are mitotic in origin, suggesting that with enough cells present, PGT results could be altered (McCoy *et al.*, 2015).

Ideally, one should biopsy from the polar, mid and mural trophectoderm from a single blastocyst; however, this was not possible because these were patients undergoing IVF and not blastocysts donated to research. Northrop *et al.* (2010) examined three separate trophectoderm sections from the same blastocyst and demonstrated a concordance rate of 80% (40/50 blastocysts), but this study did not record the location of the trophectoderm samples in relation to the ICM. Another limitation was performing AH on day 3. AH allows for premature hatching which may disrupt the true chromosomal makeup within the embryo or influence cell distribution. It is possible that the heat generated from the laser could disrupt cell junctions and affect further embryological development, possibly allowing for the premature expulsion of cells (White *et al.*, 2018). However, research in the mouse demonstrates that embryos hatch equally from the polar, mid and mural trophectoderm, suggesting a limited effect on the AH procedure (Schimmel *et al.*, 2014). Our data are similar, for the 166 blastocysts there was no difference between which area (polar, mid, or mural) hatched out of the blastocyst, 37.8%, 30.7% and 31.5%, respectively ($P$ = NS). Further research is needed whereby AH is not performed and blastocysts are not exposed to the laser until biopsy, at day 5 or 6.

Most of the research with mosaicism at the blastocyst stage deals with the reanalysis of array comparative genomic hybridization samples or the mixing of known cell lines to determine the per cent mosaicism present in the entire blastocyst (Ruttanajit *et al.*, 2016). The only way we can understand aneuploidy and blastocyst morphology is to isolate individual cells within the blastocyst and effectively 'map' the cells, creating a virtual image of the blastocyst (Taylor *et al.*, 2016). This study has already been performed and,

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although the cost was prohibitive, larger studies are certainly warranted.

In conclusion, these data do not support the hypothesis that aneuploidy is evenly distributed throughout the trophectoderm. This study adds to the pool of data that may help patients and clinicians understand why some embryos diagnosed as 'euploid' fail to implant. Further research is needed to better understand aneuploidy at the blastocyst stage and its clinical consequences.

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**Conflicts of interest.** None.

**Ethical standards.** The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

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