

**Commentary title:**

Current status and future prospects of non-invasive preimplantation genetic testing for aneuploidy

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**Commentary main:**

Preimplantation genetic testing for aneuploidy (PGT-A) aims to assist in the identification of euploid embryos generated using assisted reproductive techniques (ARTs), allowing them to be prioritised for transfer, in preference to sibling embryos with cells affected by detrimental chromosomal abnormalities. The selection of chromosomally normal embryos should, theoretically, lead to a reduction in the risks of miscarriage, aneuploid conception and implantation failure. Any strategy improving the likelihood that the embryo selected for transfer is viable should reduce the time required to achieve a pregnancy using ART. In most cases, PGT-A begins with the biopsy of ~5 cells from the external trophoctoderm (TE) layer of blastocyst stage embryos – cells which will ultimately form the extraembryonic tissues, such as the placenta. The removal of a small number of cells from the TE is thought to have a lesser impact on embryo development, compared to the sampling of one or two blastomeres at the cleavage stage. The biopsied TE cells are subjected to genetic analysis with the use comprehensive molecular cytogenetic methodologies, such as next generation sequencing (NGS), while the blastocysts from which they were derived are typically vitrified until testing is complete. Any embryos determined to contain euploid cells are warmed and transferred in subsequent cycles.

One of the main challenges associated with modern PGT-A methods, involving a specimen comprised of several cells, analysed using NGS, is the detection of a mixture of normal and aneuploid cells (mosaicism) in the biopsy specimen. A second issue is the biopsy procedure itself. Specifically, the way that the biopsy procedure is currently carried out has not been standardised and significant variation in technique exists among IVF laboratories. The method of breaching the zona pelucida (mechanical or laser), the way that the sample is separated from the rest of the embryo, and the number of cells biopsied from the TE all vary.

It is highly likely that the biopsy of larger numbers of cells from the TE increases the risk or impairing the embryo's implantation potential. Furthermore, even if efforts are made to restrict the size of the biopsy specimen, it is not possible to precisely control the number of cells taken. Another consideration with respect to embryo biopsy is the cost that it entails for the patient, which in some cases can be more expensive than the genetic component of the PGT-A test.

Several recent investigations reported on the presence of embryonic DNA either in the liquid filling the blastocoel cavity of blastocyst stage embryos (blastocoel fluid - BF), or in the medium drop into which the embryos were cultured (1, 2, 3). The mechanism of embryonic DNA expulsion either into the BF or the culture medium has not been determined, although it has been hypothesised that it might be released from cells undergoing apoptosis or some other process that results in a loss of membrane integrity. Investigations have attempted to process the DNA found either in the BF or the spent culture medium (SCM) in a similar way to that of a biopsied TE sample, in an effort to obtain information on the embryo's cytogenetic constitution without the need of a biopsy. Such a non-invasive PGT-A approach would have various advantages over the current strategies based upon embryo biopsy, including the elimination of a costly micromanipulation procedure, and the avoidance of risks associated with the removal of cells.

Ho and colleagues (4) carried out a prospective study aiming to assess the accuracy of identifying embryonic cell free DNA (cfDNA) in SCM, and examine whether factors such as assisted hatching (AH) or embryo morphology would affect the amounts of cfDNA available. The study included a pilot phase involving the analysis of SCM and previously cryopreserved embryos donated for research, and a clinical phase involving the analysis of patient samples. A modified whole genome amplification (WGA) followed by NGS were employed to investigate embryonic samples and cfDNA in SCM obtained on days-3 and -5, post-

fertilization. Sensitivity, specificity, positive predictive value and negative predictive value were found to be better after day-5 cfDNA analysis than day-3, while AH and embryo morphology did not seem to have a significant influence on cfDNA concentration in the SCM. However, the reported ploidy concordance rates between SCM samples and whole embryos or TE biopsies were relatively poor (56.3% and 65% respectively), leading the authors to conclude that non-invasive PGT-A requires further technical optimisation.

A better concordance rate of 84% was reported in a similar investigation carried out by Xu et al. (2). There were several methodological differences between the two studies, including the use of 2 different WGA methods (Ho et al. used PicoPlex, whereas Xu et al., used multiple annealing and looping-based amplification cycles or MALBAC). Moreover, all embryos assessed by Xu et al. (2) were initially vitrified at the cleavage stage. Conversely, Ho et al. (4) assessed SCM from embryos vitrified at the zygote stage during the pilot phase of their study, and SCM from fresh embryos cultured in a continuous system during the clinical phase. Ho et al. (4) speculated that the poor concordance rates could be attributed to the presence of maternal cumulus cells, something that was also observed by Vera-Rodriguez and colleagues (3). Mosaicism (a biological factor) or amplification artefacts (a technical consideration) were also proposed as possibilities that could might contribute to diagnostic errors.

Considering all results reported to date, assessing the presence of cfDNA in the SCM, including the studies discussed herein, it is evident that non-invasive PGT-A applied to culture media droplets is not ready for clinical application at the current time. It is unclear whether the problems can be solved through technical innovation or whether cfDNA existing in SCM is fundamentally unsuitable for genetic diagnosis. If the lack of concordance between whole embryo and SCM samples is related to the nature of the cfDNA, could alternative sources of embryonic DNA yield better results. Magli and colleagues (1) in their study

examining cfDNA in BF aspirated from blastocysts, reported a remarkably high ploidy concordance of 97% with respect to TE samples from the same embryos. A standard WGA without any specific modifications was employed during that study, followed by aCGH. Sensitivity and specificity were also very high. The aspiration of BF from inside the embryos may have had the added advantage of alleviating the risk of extraneous DNA contamination (e.g. derived from cumulus cells or other non-embryonic sources). In many clinics, removal of the BF is already routinely performed as part of blastocyst cryopreservation procedures, in order to reduce the risk of ice crystal formation, and appears to be harmless. While Magli's results are encouraging, it is of note that several other groups who have attempted BF analyses have not achieved such good results, suggesting that there are important subtleties of technique that may be critical for obtaining high rates of cfDNA amplification and accuracy.

There are various unanswered questions which need to be addressed before a wider clinical application of non-invasive PGT-A can be contemplated. Perhaps the most critical is the need to understand the degree to which the cytogenetic status of cfDNA is representative of the chromosome constitution of the corresponding embryo. The studies published to date vary widely in the reported concordance rates of cfDNA versus cellular material (entire whole embryos or the biopsied TE samples). It is also unclear which is most appropriate stage for the collection of SCM drops. Most investigations have used a sequential culture system and examined SCM drops in which the embryos were incubated between days-3 and -5. There is less data from strategies where SCM drops were obtained after continuous embryo culture from day-1 to day-5 or -6 of preimplantation development (5), but what limited data exists is suggestive of higher cfDNA concentrations in comparison with sequential culture. Moreover, the reason why embryonic DNA is expelled into the surrounding environment is unknown. If the cfDNA is indeed the remnant of cells that have been destroyed by apoptosis or other processes of cell death, it is important to understand the underlying reason for the

cell's demise. In blastocysts, it is possible that apoptotic pathways are activated so as to eliminate aneuploid cells in mosaic embryos composed of a mixture of diploid and aneuploid cells. If this is the case, examining cfDNA could risk false-positive detection of aneuploidies, which the embryo has actually succeeded in eliminating. Another question is whether or not the DNA is truly 'cell free' or whether whole embryonic cells are sometimes shed into the surrounding medium or blastocoel cavity. Again, this could represent a mechanism for the disposal of abnormal cells. Non-invasive PGT-A could also be associated with additional costs to the patient. Specifically, assessment of cfDNA in SCM would require embryos to be cultured in separate drops, rather than together. This may result in a loss of some benefits believed to be associated with group culture and will lead to an increase in the total volume of culture medium required per patient, as well as the number of petri dishes and incubators used. Hence it is possible that some of the costs of biopsy may ultimately be replaced with other costs to the patient, associated with IVF consumables.

In summary, the Ho et al. (4) study provided useful data concerning the possibility of performing non-invasive PGT-A at the cleavage and blastocyst stages. It was clear from the results of this study that cfDNA can be detected in the SCM, but before clinical application can be contemplated, it is essential to improve the concordance with cytogenetic results obtained from the corresponding embryos. Non-invasive PGT-A remains an exciting possibility, which holds great promise for the future, but there are still many factors to be considered prior to its wider implementation.

## **References**

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