

## **Listing title**

Non-invasive preimplantation genetic testing (NI-PGT): the next revolution in reproductive genetics?

## **Running title**

Non-invasive preimplantation genetic testing

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## **Key words**

Preimplantation genetic testing; preimplantation genetic diagnosis; preimplantation genetic screening; embryo biopsy; blastocentesis; blastocoel fluid; trophectoderm biopsy; cell-free DNA; medium; aneuploidy

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## **Manuscript type**

Comprehensive literature review article

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## **Abstract**

### ***Background:***

Preimplantation genetic testing (PGT) encompasses methods that allow embryos to be tested for severe inherited conditions or for chromosome abnormalities, relevant to embryo health and viability. In order to obtain embryonic genetic material for analysis, a biopsy is required, involving the removal of one or more cells. This invasive procedure greatly increases the costs of PGT and there have been concerns that embryo viability could be compromised in some cases. The recent discovery of DNA within the blastocoele fluid (BF) of blastocysts, and in spent embryo culture media (SCM), has led to interest in the development of non-invasive methods of PGT (NI-PGT).

### ***Objective and rationale:***

This review evaluates the current scientific evidence regarding non-invasive genetic assessment of preimplantation embryos. The success of different PGT methodologies in collecting and analyzing extra-embryonic DNA is evaluated, and consideration is given to the potential biological and technical hindrances to obtaining a reliable clinical diagnosis.

### ***Search methods:***

Original research and review papers concerning NI-PGT were sourced by searching PubMed and Google Scholar databases until January 2019. Searches comprised the keywords: ‘non-invasive’; ‘cell-free DNA’; ‘blastocentesis’; ‘blastocoel fluid’; ‘spent culture media’; ‘embryo culture medium’; ‘preimplantation genetic testing’; ‘preimplantation genetic diagnosis’; ‘preimplantation genetic screening’; ‘aneuploidy’.

### ***Outcomes:***

Embryonic DNA is frequently detectable in BF and SCM of embryos produced during IVF treatment. Initial studies have achieved some success when performing cytogenetic and molecular genetic analysis. However, in many cases, the efficiency has been restricted by technical complications associated with the low quantity and quality of the DNA. Reported levels of ploidy agreement between SCM/BF samples and biopsied embryonic cells vary widely. In some cases, a discrepancy with respect to cytogenetic data obtained after trophectoderm biopsy may be attributable to embryonic mosaicism or DNA contamination (usually of maternal origin). Some research indicates that aneuploid cells are preferentially

eliminated from the embryo, suggesting that their DNA might be overrepresented in SCM and BF samples - a hypothesis that requires further investigation.

### ***Wider implications:***

Available data suggest that BF and SCM samples frequently provide DNA templates suitable for genetic analyses, offering a potential means of PGT that is less expensive than traditional methods, requires less micromanipulation skill and poses a lower risk to embryos. Critically, DNA isolation and amplification protocols must be optimized to reproducibly obtain an accurate clinical diagnosis, whilst minimizing the impact of confounding factors such as contamination. Further investigations are required to understand the mechanisms underlying the release of embryonic DNA and to determine the extent to which this material reflects the true genetic status of the corresponding embryo. Currently, the clinical potential of NI-PGT remains unknown.

## **Introduction**

The ultimate goal of *in vitro* fertilization (IVF) is to achieve a healthy birth, ideally from a single transferred embryo. Over the last thirty years, a variety of methods have been developed that allow the genetic assessment of preimplantation embryos. These have been employed either to avoid the transmission of a single gene disorder (preimplantation genetic testing for monogenic disease; PGT-M) or, more controversially, in an effort to aid the selection of viable embryos for uterine transfer - distinguishing chromosomally normal embryos, considered to have high developmental potential, from their compromised aneuploid siblings (preimplantation genetic testing for aneuploidy; PGT-A).

In order to infer the genetic status of the embryo, all past and current PGT methods have relied upon the sampling of genetic material from the oocytes/zygotes (via removal of polar bodies) or embryos (involving biopsy of blastomeres or cells of the trophectoderm) (Dokras *et al.*, 1990; Handyside *et al.*, 1990; Kokkali *et al.*, 2005; Verlinsky *et al.*, 1990). However, the micromanipulation techniques required for biopsy are invasive and are not

entirely without risk (Scott, Upham, Forman, Zhao, *et al.*, 2013). Additionally, such methods carry costs in terms of the need to purchase and maintain specialist equipment (principally a laser, used to breach the zona pellucida prior to removal of cells) and the staffing costs associated with highly skilled embryologists investing significant amounts of time conducting biopsy procedures. The need to find funds for equipment and train staff in biopsy techniques also means that some clinics may be unable to initiate a PGT-A program, or there may be substantial delays as well as a limit to the number of procedures that can be carried out each day. With ever-declining costs of genetic testing, the fee for embryo biopsy has become the single greatest contributor to the total cost of PGT in some clinics.

Interestingly, substantial variation exists between the clinical outcomes of PGT cycles in different clinics (Gutiérrez-Mateo *et al.*, 2009). The disparity in the rates of diagnosis and pregnancy that are sometimes observed are unlikely to be entirely explained by differences in patient populations. Embryo biopsy techniques have never been standardized and it is reasonable to speculate that a portion of the inter-clinic and inter-biopsy practitioner variation in results following PGT may be a downstream consequence of divergent methodologies, some of which may be superior to others.

Given the challenges associated with invasive embryo biopsy, it is not surprising that the recent discovery of DNA in embryo culture media, and also in the fluid filling the blastocoel cavity of blastocysts, has ignited excitement about the possibility of non-invasive PGT (NI-PGT) (Palini *et al.*, 2013; Stigliani *et al.*, 2013). Ongoing studies are now seeking to verify whether this DNA represents a reliable source of information about the genetic status of the embryo. If accurate PGT proves to be possible, less invasive approaches based upon ‘extra-embryonic’ DNA may provide a simpler, safer and more consistent approach to PGT. The costs of PGT would likely be reduced and bottlenecks associated with logistics, training and financing of equipment could be eliminated, thus improving access to patients.

Thus far, published results regarding the capacity of NI-PGT protocols to consistently provide accurate clinical diagnostic information have been fragmentary and sometimes contradictory. Accordingly, this review aims to critically assess the current evidence base regarding the nature of the extra-embryonic DNA and its clinical potential in the context of PGT.

### **Traditional invasive biopsy techniques**

It is fair to say that PGT methods based upon the biopsy of polar bodies, blastomeres or trophectoderm cells have been highly successful, helping thousands of families achieve healthy pregnancies (De Rycke *et al.*, 2017). The first polar body (PB) is extruded from mature oocytes around the time of ovulation, while the second PB appears after fertilization. The PBs are considered by-products of meiosis - receptacles for the chromosomes discarded by the oocyte as it becomes haploid - and are destined to degenerate. As they have no apparent role in subsequent development, nor are an integral part of the embryo, the removal of both PBs for the purposes of PGT is sometimes considered less invasive than approaches involving the biopsy of cells at later embryonic stages (Montag *et al.*, 2009). An obvious limitation of PB analysis is that it provides no information on the paternal contribution to the genetics of the embryo. Additionally, it is relatively labor intensive due to the need to biopsy and analyze all of the mature oocytes produced, even though many of the resulting embryos will ultimately arrest in culture. Increased rates of embryo fragmentation and developmental arrest have been reported following PB biopsy, suggesting that even this seemingly minimally-invasive manipulation may negatively impact embryo viability (Levin *et al.*, 2012).

For much of the history of PGT, the dominant method of biopsy involved removal of 1-2 blastomeres at the cleavage stage – most often conducted on day-3, when the embryo is typically composed of approximately 6-10 cells. However, this strategy has several drawbacks.

169 Firstly, chromosomal mosaicism which is common throughout preimplantation development,  
170 is at its highest frequency at the cleavage stage, potentially leading to errors when conducting  
171 PGT-A (Fragouli *et al.*, 2019). Secondly, the analysis of a single cell carries with it a high risk  
172 of genotyping error at individual sites during PGT-M cycles due to the phenomenon of allele  
173 dropout (ADO) – the failure of one of the two alleles present in a heterozygous cell to amplify  
174 following PCR. Typically 5-10% of single-cell amplifications experience ADO (Piyamongkol,  
175 2003). This problem has necessitated the development of more complex, redundant diagnostic  
176 tests, combining mutation detection with the analysis of multiple informative linked  
177 polymorphisms.

178 Cells of the cleavage stage human embryo are typically considered to be totipotent, and  
179 therefore embryos at this early stage are expected to display a high degree of developmental  
180 plasticity and be able to tolerate the removal of cells. However, emerging evidence suggests a  
181 degree of cell-fate specification as early as the 4-cell stage in mice (Goolam *et al.*, 2016). If  
182 specification mechanisms occur equally early in human embryos, it is possible that the removal  
183 of cells destined to contribute to the fetus during cleavage stage biopsy could harm the embryo.  
184 In accordance with this hypothesis, several studies have reported that blastomere biopsy has  
185 the potential to impair embryo viability and alter preimplantation morphology (Bar-El *et al.*,  
186 2016; Cohen *et al.*, 2007; Kirkegaard *et al.*, 2012). Most convincingly, a paired randomised  
187 controlled trial (RCT) reported a 39% relative reduction in implantation rate following single  
188 blastomere biopsy, compared to controls (Scott, Upham, Forman, Zhao *et al.*, 2013).

189 In terms of the long term health of individuals produced from embryos that underwent  
190 cleavage stage biopsy, follow-up data is sparse although generally reassuring (Kuiper *et al.*,  
191 2018; Sacks *et al.*, 2016). Nevertheless, it may be worth noting that an enhanced risk of  
192 neurodegenerative disorders, a higher body weight, and epigenetic modifications have been  
193 reported in mice produced following blastomere biopsy (Yu *et al.*, 2009; Zhao *et al.*, 2013).

An important innovation was the introduction of blastocyst biopsy, most often performed on day-5 post-fertilization (Kokkali *et al.*, 2005). Blastocysts are more resilient than embryos at earlier developmental stages, with a greater capacity to tolerate micromanipulation. Typically, 5-10 trophoctoderm cells are biopsied, allowing retrieval of more genetic material despite removing an overall lower percentage of embryonic mass relative to cleavage stage biopsy. The increased amount of DNA reduces the risk of amplification failure and ADO, whilst, in the context of PGT-A, providing an opportunity to detect some instances of chromosomal mosaicism (Chang *et al.*, 2013; Fragouli *et al.*, 2017; Kokkali *et al.*, 2007; Maxwell *et al.*, 2016). Reports that blastocyst biopsy poses less risk to the embryo than removal of blastomeres resulted in a dramatic uptake of the procedure, and today the vast majority of embryo biopsies for PGT are conducted using this method (Glujovsky *et al.*, 2016; McArthur *et al.*, 2008; Scott, Upham, Forman, Hong, *et al.*, 2013).

However, with the rush towards blastocyst biopsy, it is possible that some potential risks associated with this strategy have been overlooked. Biopsy protocols remain technically challenging, invasive procedures, which could conceivably impact embryo viability if not performed correctly. For example, it has been suggested that trophoctoderm biopsies involving the removal of relatively large numbers of cells may be associated with lower birth rates than those where fewer cells are taken (Neal *et al.*, 2017). It is anticipated that less invasive means of acquiring DNA for PGT should be associated with minimal risk to embryos. Moreover, such methods could potentially permit the analysis of embryos of poor morphological grade which are often considered unsuitable for biopsy and are sometimes discarded without ever undergoing genetic analysis.

#### **‘Blastocentesis’ – Sampling and analysis of DNA in the blastocoel cavity**

During blastocyst formation, embryonic cells differentiate into the inner cell mass



(ICM) and trophectoderm (TE) lineages. This occurs coincident with ion gradient formation from Na<sup>+</sup>/K-ATPase activity across the epithelium, promoting passage of water through transmembrane channels, leading to the formation of a fluid-filled ‘blastocoel’ cavity (Watson and Kidder, 1988). Subsequent zona pellucida thinning and hatching prepares the embryo for uterine implantation (Cohen *et al.*, 1990). The discovery of DNA suitable for amplification and genetic testing in the BF (BF-DNA) was first reported by Palini *et al.* (2013) and resulted in great interest concerning the potential for a new era of minimally-invasive preimplantation genetic tests. During the BF sampling procedure, sometimes termed ‘blastocentesis’, an ICSI pipette pierces through the trophectoderm layer on the opposing side of the embryo from the ICM, and the fluid is carefully aspirated, leaving the embryo fully collapsed (Magli *et al.*, 2016; Poli *et al.*, 2015).

The loss of BF should not, in theory, be detrimental to the embryo. Dynamic collapse and re-expansion of the cavity is a phenomenon frequently observed during *in vitro* blastocyst culture prior to hatching (Bodri *et al.*, 2016; Marcos *et al.*, 2015). In addition, the deliberate collapse of the blastocoel cavity is performed during many blastocyst vitrification procedures. The water content of BF reduces embryo permeability to cryoprotectants, increasing the risk of ice-crystal formation and cellular damage during cryopreservation, hence emptying of the cavity is considered advantageous (Mukaida *et al.*, 2006; Vanderzwalmen *et al.*, 2002). It is certainly true that a degree of skill is required in order to sample BF. Nonetheless, avoiding the additional effort of learning embryo biopsy techniques could help to reduce an important bottleneck to clinical application associated with training. Additionally, since removal of BF is already a routine procedure in many clinics, blastocentesis may represent relatively little additional effort or expense for clinics - especially in ‘freeze-all’ cycles where all embryos undergo vitrification.

#### **PGT-M using blastocentesis**

There are two major prerequisite requirements for clinical application of PGT using minimally-invasive sources of DNA. Firstly, it must be possible to consistently isolate and analyse the DNA, to avoid the possibility of failing to obtain a clinical diagnosis. Secondly, the DNA must be shown to be representative of the developing embryo to ensure that the diagnosis is accurate. Regarding the former, attempts to perform PGT have encountered significant difficulties in isolating and amplifying DNA retrieved using blastocentesis. This is thought to relate to the low quantity and poor quality of BF-DNA, which has been estimated at <10pg and is believed to be degraded in nature (Gianaroli *et al.*, 2014; Palini *et al.*, 2013; Zhang, Li, *et al.*, 2016).

The first study to consider the theoretical possibility of using BF for PGT evaluated the potential for determining the sex of embryos, a strategy allowing X-linked conditions to be avoided by ensuring that only female embryos are transferred (Palini *et al.*, 2013). Quantitative PCR (qPCR) was employed for the amplification of a multicopy Y-chromosome gene (*TSPY1*) and an autosomal control gene (*TBC1D3*) on chromosome 17. Of 29 BF samples analyzed, 89.7% presented a detectable *TBC1D3* product and *TSPY1* amplification was observed in 65.4% of these, suggesting that the corresponding embryos were male. Whilst the sex ratio bias might be explained by the small sample size, this was not confirmed using a standard biopsy-based method. Moreover, although the targeting of multicopy genes for amplification may potentially enhance the sensitivity of DNA detection, this is not applicable to PGT for disorders deriving from single-copy genes.

To enhance template copy numbers for subsequent analyses of individual loci or chromosome copy number analysis, a number of whole genome amplification (WGA) protocols have been applied to BF-DNA. Varying efficiencies have been reported between studies, with amplification successful in 34.8-87.5% of BF samples studied (Capalbo *et al.*, 2018; Gianaroli *et al.*, 2014; Magli *et al.*, 2016, 2018; Palini *et al.*, 2013; Tobler *et al.*, 2015;

Tšuiiko *et al.*, 2018; Zhang, Li, *et al.*, 2016). The genomic coverage of WGA products produced using the PicoPLEX (NEB) kit was evaluated by Zhang, Li, *et al.*, (2016) in a small, but possibly indicative, study. Whilst WGA was successful in only 72.7% of BF samples (8/11) compared to 100% of blastomere biopsies (11/11), next-generation sequencing (NGS) analysis of the products revealed that a similar proportion of the genome had amplified in paired samples - albeit providing a slightly lower sequencing depth in some regions in BF samples. Likewise, investigation of chromosome 1 at finer resolution, as a representative genomic paradigm, revealed similar localised ‘islands’ of sequencing read coverage between both sources of DNA – alike in depth and location. Therefore, it appears that on the occasions when BF contains DNA suitable for amplification, a full representation of the embryonic genome can be obtained, equivalent to that which would be obtained from a cell biopsy. Additional PCR of 10 disease-associated genes yielded successful amplification in 84% of BF samples (n=5). Subsequent proof-of-principle Sanger sequencing of *TCIRG1* and *SCN5A* amplicons, encompassing mutation sites associated with osteopetrosis and long QT syndrome type 3, respectively, revealed full concordance with results from blastomere biopsies in the single sample tested.

Similar results were observed by Galluzzi *et al.* (2015), who further evaluated the potential to detect point mutations using BF-derived WGA products. PCR was used to amplify a 109bp region of the *MTHFR* gene encompassing a polymorphism (C677T) potentially associated with unexplained recurrent pregnancy loss (Cao *et al.*, 2013). However, WGA-PCR amplification rates were only 44.4% (in 9 samples) and only one paired trophoctoderm biopsy was available to validate concordance. The data reported by both these studies suggest that WGA products generated from blastocentesis samples display similar gene coverage to blastomere biopsies, are amenable to downstream PCR analysis for disease-associated genes and may represent the embryos’ genetics at the nucleotide level. However, the sample sizes

were insufficient to draw definitive conclusions and the analyses were restricted by the relatively low amplification rates achieved relative to embryo biopsies.

Whilst it could be argued that the relatively disappointing amplification rates in both studies could be associated with the use of the same WGA technology (PicoPLEX, Rubicon Genomics), PCR amplification rates were no better in a report following a multiple displacement amplification (MDA) based WGA approach (REPLI-g, Qiagen) applied to seven paired BF and trophectoderm biopsies (Shangguan *et al.*, 2017). An attempt was made to amplify regions of the spinal muscular atrophy, phenylketonuria, *SRY* and  *$\beta$ -actin* genes from WGA products. However, for the autosomal genes, amplification was achieved in only 42.9-71.4% of BF samples, compared to 100% of trophectoderm biopsies. Nevertheless, the 6 BF samples from which amplicon sequencing data was successfully obtained, shared identical nucleotide sequences with TE counterparts.

The difficulties in amplification seen in these three studies are consistent with the notion that DNA within the blastocoel fluid is restricted in quantity and likely to be somewhat degraded. Collectively, the investigations indicate that BF-DNA is representative of the embryo at the nucleotide level and potentially an appropriate source of material for use in single-gene PGT, provided that amplification protocols can be improved. However, more recently, Capalbo *et al.* (2018) reported much lower concordance between BF-DNA and trophectoderm biopsies when performing PGT-M. A validated qPCR-based methodology (TaqMan Genotype Assays), specific to each couple undergoing PGT-M, was used to analyze the mutation site responsible for the disorder and linked single nucleotide polymorphisms (SNPs) without prior WGA. In a prospective trial using trophectoderm biopsies, this methodology previously achieved successful amplification in all samples, 0% ADO, and over 99% genotype consistency with reference laboratory results (Zimmerman *et al.*, 2016). However, the embryo haplotype obtained from the BF was only fully concordant with the

trophectoderm biopsy in 2.9% of 69 paired samples. Moreover, when 347 loci were analyzed in 69 BF samples, only 27.4% amplified; compared to 100% of the 405 loci analyzed in 80 TE samples. Interestingly, ADO occurred predominantly in alleles of paternal origin (31.6%) rather than those derived from the mother (14.7%), perhaps indicating that the lower rates of concordance in this study stem from the presence of maternal DNA contamination from cumulus cells or polar bodies. These figures compare to the TE control, in which ADO occurred at only a single locus of one embryo (0.25% of 405 loci).

In conclusion, studies attempting to evaluate the suitability of BF-DNA for PGT-M have not yet been able to demonstrate sufficient amplification rates in an adequate number of samples, for clinical application to be considered (Table 1). PCR amplification efficiencies following WGA are consistently lower than that reliably achieved following cellular biopsy and consequently, the risk that an embryo will remain undiagnosed following PGT-M is elevated for these BF strategies. Moreover, as ADO rates are enhanced when DNA is degraded, there may be an increased risk that diagnoses using BF-DNA could be compromised (Huang *et al.*, 2015; Piyamongkol, 2003). Future approaches may avoid some of these technical issues by employing novel amplification technologies adapted to degraded DNA or by using more sensitive DNA detection technologies such as NGS, digital PCR (dPCR) and pyrosequencing. Redundant diagnostics, utilizing multiple informative linked polymorphisms, would also be important to reduce the risk of misdiagnosis due to ADO. It is also necessary to quantify the incidence of contamination of BF samples with non-embryonic DNA, as data concerning this question is currently lacking.

#### **PGT-A using blastocentesis**

PGT-A can be performed using various comprehensive chromosome screening (CCS) technologies including qPCR, array comparative genomic hybridization (aCGH), SNP arrays, and NGS (Munné and Wells, 2017). Alongside their PGT effort for X-linked disorders, Palini

*et al.* were the first to demonstrate that cytogenetic assessments might be possible using BF-DNA. Following WGA (REPLI-g, Qiagen), aCGH yielded aneuploidy results for two samples. However, the accuracy of these results could not be verified since the concordance with the corresponding embryos was not determined. Several subsequent studies have expanded this work, investigating the reliability of BF-DNA as a template for PGT-A, and reaching conflicting conclusions (Table 2).

Gianaroli *et al.* (2014) used WGA (SurePlex, Illumina) and aCGH (24Sure, Illumina) to investigate karyotype concordance of BF with cells of 51 supernumerary embryos obtained from 17 couples undergoing fertility treatment. A cytogenetic evaluation was performed using either polar bodies (n=37) or blastomere biopsy (n=14), before subsequent additional analysis of the BF and biopsied trophoctoderm cells at the blastocyst stage. Of the 76.5% of BF-DNA samples which successfully amplified following WGA, chromosome concordance rates per single chromosome were 93.5%, 94.0% and 96.6% with respect to the polar body, blastomere and trophoctoderm biopsies, respectively. Moreover, in terms of overall ploidy (i.e. classification of the embryo as normal or aneuploid), the concordance was 93.3%, 100.0% and 97.4%.

A degree of discordance is expected between different types of biopsy specimen. Paternal-derived errors would elicit discordance between the PBs and embryo biopsy/BF-DNA, whilst differences with respect to embryonic cells may result from mitotic chromosome segregation errors, producing mosaicism. However, if blastocentesis is to be considered for routine clinical use, it is important to establish whether the BF-DNA is any less concordant with the remaining embryo than a conventional TE biopsy. In Gianaroli's study, concordance between TE and blastomere biopsies of the same embryo were similar to those observed for the BF-DNA and the embryo (100% concordance in ploidy status and 98.4% for individual chromosomes). Thus, these data indicate that most BF-DNA samples that yield a result provide

an accurate representation of the cytogenetic status of the embryo, providing promise for the clinical application of blastocentesis.

The same group replicated these promising results in an analysis of 116 blastocysts derived from 51 couples undergoing aCGH for PGT-A (Magli *et al.*, 2016). As before, PB or blastomere biopsy was followed by subsequent blastocentesis and trophoctoderm biopsy. WGA was successful in 82% of BF samples, which is still inferior to amplification rates seen in trophoctoderm biopsy specimens and arguably too low for clinical use, but nonetheless represents one of the best series of results reported thus far for BF. An analysis was performed in the 70 amplified samples in which informative chromosome copy numbers were also available for biopsy specimens. Concordance rates of BF-DNA, with respect to PBs, blastomeres and trophoctoderm biopsies, were 97.9%, 97.7% and 98.4% at the chromosomal level and 94.0%, 95.0% and 97.1% in terms of overall ploidy. Promisingly, in comparison with results obtained using trophoctoderm biopsy, which despite some deficiencies represents today's current clinical gold standard for PGT-A, sensitivity and specificity rates with regards to ploidy status were 0.98 and 0.93, respectively.

The reproducibility of Gianaroli and colleagues' method in obtaining such results was shown again recently in an analysis of paired BF and trophoctoderm biopsies from 256 blastocysts (Magli *et al.*, 2018). Using the same PGT-A strategy, successful amplification occurred in 71% of BF samples; of which 87% provided an informative chromosome copy number result. As in previous research, rates of ploidy concordance and concordance per single chromosome were 93.6% and 96%, respectively, whilst full chromosome concordance rates were slightly lower (66.3%).

Although this data concerning the ability to obtain an accurate cytogenetic evaluation using blastocentesis has been encouraging, it has been disputed by three other groups. In a cohort of 23 paired BF and TE biopsies from 12 couples, Capalbo *et al.* (2018) achieved

successful WGA in only 34.8% of BF samples. Furthermore, of the nine samples for which chromosome copy number data was obtained, only 37.5% displayed a karyotype that was fully concordant with the corresponding trophectoderm biopsy – less than half the rate reported by Gianaroli and colleagues. However, it is noteworthy that TE biopsies were subject to qPCR-based aneuploidy testing, whilst BF specimens were processed by WGA (SurePlex) and NGS testing (VeriSeq, Illumina). The use of a single PGT-A technology, ideally NGS-based, for both sample types would have been preferable from an experimental perspective.

Similar data were reported by Tobler *et al.* (2015), who performed PGT-A using BF-DNA obtained from 96 embryos deemed unsuitable for clinical treatment; some vitrified-warmed at the blastocyst stage, and others at cleavage stage, which were subsequently cultured to the blastocyst stage for blastocentesis. Collectively, molecular karyotypes were successfully obtained in 63% of BF-DNA samples, analysis of which revealed concordance rates with the whole embryo (ICM and TE cells) of only 62% for overall ploidy status, and 48% for specific karyotype. These data collectively translated to sensitivities and specificities of 0.88 and 0.55, respectively – diagnostic accuracies unacceptable for clinical use. Thus, despite adopting the same blastocentesis, WGA (SurePlex, Illumina) and aCGH (24-sure, Illumina) methodologies as Gianaroli, Magli and colleagues, extreme inconsistencies in concordance between BF-DNA and biopsy samples were observed between the groups.

It is possible that the discrepancies in levels of concordance amongst studies may result from inter-laboratory differences in the levels of mosaicism in the cohort of embryos analyzed. The thawed cleavage stage embryos used by Tobler and colleagues (n=29), provided a single blastomere biopsy available for analysis, which revealed that only 20.7% of all blastomere biopsy, BF-DNA and ICM-TE results were concordant and euploid. Importantly, higher rates of concordance were observed between BF-DNA and blastomere biopsy, than between blastomere biopsy and ICM/TE. This may indicate that mosaic embryos contain a mechanism



of euploidization, involving the preferential elimination of aneuploid cells into the BF during blastulation, to maximize embryo viability. In accordance with this hypothesis, 100% of the 9 cleavage stage embryos associated with a euploid blastomere biopsy remained euploid at the blastocyst stage, as determined by the sequential trophectoderm biopsy. Notably, three of these embryos displayed an aneuploid BF-DNA result. Moreover, 70% of the 20 embryos which displayed an aneuploid blastomere biopsy appeared to normalize, producing euploid trophectoderm biopsies; of which 86% had an aneuploid paired BF-DNA result.

Further data in agreement with this hypothesis were reported by Tšuiiko *et al.* (2018) who examined concordance between paired BF, ICM and TE samples, which had been dissected from 16 frozen-thawed blastocysts. An advantage of this study was that all TE and ICM cells were available for analysis, rather than only a single biopsy. This is significant because a biopsy taken from a mosaic embryo, may not fully represent the karyotype of the remaining cells of the embryo, and thus incorrectly suggest that the BF-DNA result is erroneous. Following WGA (PicoPLEX, Rubicon Genomics), an NGS platform (VeriSeq, Illumina) was adopted for aneuploidy detection. The ability to obtain detectable DNA and a chromosome copy number assignment from BF were similar to other methods (87.4% and 62.5% of samples, respectively). In contrast, the same technique yielded chromosome copy number results in 87.5% of TE and ICM samples.

In three paired samples, reciprocal aneuploidies (a loss or gain of a chromosome in one sample and a respective gain or loss in the corresponding sample) were observed between BF-DNA and embryo biopsies, indicating that chromosome nondisjunction had occurred during mitosis. Unlike in the study by Tobler and colleagues, this investigation considered reciprocal aneuploidies as concordant, since they are presumed to have a shared mitotic origin. Even so, full chromosome concordance rates were only 40.0% between BF-DNA and ICM samples, and similarly 40.0% between BF-DNA and TE samples. These were much lower than within the

blastocyst itself: an 85.7% concordance rate was observed between ICM and TE samples. Furthermore, results indicative of apparent mosaicism were much more prevalent in BF-DNA, and a higher number of chromosomes were affected; even when TE and ICM counterparts were chromosomally normal. These data further support the hypothesis that abnormal cells may be excluded from mosaic cleavage stage embryos, either through active or passive mechanisms, expelling some into the blastocoel during blastulation, and consequently compromising the clinical utility of BF-DNA for PGT-A.

It remains unclear why poor concordance and/or elevated rates of aneuploidy in BF were only seen in the studies by Tšuiiko, Tobler and Capalbo, and not those by Gianaroli, Magli and colleagues. It might be the case that the samples assessed by Gianaroli's group predominantly contained aneuploidies of meiotic origin, rather than mitotic. In this circumstance, all cells would be expected to contain the same karyotype and have a higher concordance between biopsies, compared to embryos containing a mixture of karyotypes following errors of mitotic origin. This is plausible as rates of mosaicism have been reported to vary between clinics, from 17 to 48% (Munné *et al.*, 1997; Sachdev *et al.*, 2016).

Differences also may relate to the quality of samples studied; the embryos used by Tšuiiko and Tobler were leftover following treatment, with Tobler's study specifically stating that they were unsuitable for clinical use. This compares to Gianaroli, Magli and colleagues, who obtained samples were obtained from patients who were currently undergoing PGT-A, and thus would likely contain some good quality embryos suitable for clinical use. Whilst Capalbo's study also used samples obtained from couples undergoing PGT-A, they observed that concordance rates were higher for samples which were of good morphology, though this was not statistically significant. It is also important to note that sample sizes in the studies by Capalbo and Tšuiiko were much smaller than those by Gianaroli, Magli and colleagues, and thus different results may have been obtained if a larger size was studied.

Discrepancies in concordance rates may also relate to differences in embryological handling between the studies. Particularly, the samples used by Gianaroli, Magli and colleagues underwent many manipulations (laser-assisted PB biopsy, assisted hatching and blastomere biopsy), whereas the other studies performed no, or comparatively fewer manipulations (Table 2). Thus, the enhanced amplification and concordance rates observed may be a result of inadvertent cell lysis or death upon manipulation, during which an increased quantity of available DNA in the blastocoel cavity for a robust and accurate analysis.

Another possibility is that some studies attempted to assign the cytogenetic status of BF samples in instances when the WGA had not provided sufficient amplification. Inadequate WGA and the amplification of highly degraded DNA samples frequently result in artefactual aneuploidies when analyzed using NGS or aCGH, and might have inflated the apparent aneuploidy level seen in BF samples. Such artefacts are not unexpected given the likely degraded nature of the DNA in the blastocoel. This is plausible considering that in the studies reporting lower levels of concordance, discordance mainly was related to increased levels of chromosomal abnormalities in the BF. It may be necessary to institute more stringent criteria for analyzing data from BF samples, perhaps leading to improved rates of concordance, but likely at the expense of fewer samples providing a result.

Differences in the CCS platform used for PGT-A analysis may also have an impact on the differences in success rates observed between studies; Tšuiiko and Capalbo adopted an NGS method, whilst the other studies used aCGH to determine chromosome copy number. Due to its comparatively high sensitivity, NGS can provide valuable information concerning embryonic mosaicism, which has been difficult to detect using previous technologies (Munné and Wells, 2017). Accordingly, it is plausible that higher rates of discordance in these studies may result from the additional analysis of chromosomal aberrations, which went undetected when aCGH was used. For example, when evaluating full chromosome concordance, Tšuiiko

and colleagues considered a case discordant when the TE/ICM displayed a 45 XY -13 karyotype and the BF displayed a karyotype of 45 XY -13, Mosaic +1 (60%), Mosaic -16 (30%), Mosaic -21 (40%). As Gianaroli, Magli and colleagues did not report any incidence of mosaicism, their technology possibly would not have detected these abnormalities and would have classified these samples as concordant. However, whilst this could have resulted in lower rates of full chromosome concordance, it does not explain the lower rates of ploidy concordance observed in the study by Tšuiiko. Further studies adopting an NGS approach to analyze whole dissected embryos are required to shed further light on how mosaicism may influence data interpretation and critically whether BF-DNA comprises clinically useful information.

#### **Technical caveats to blastocentesis**

Whilst blastocentesis is an appealing concept, difficulties achieving consistent amplification of BF-DNA has limited research to date; reducing sample sizes, leaving studies underpowered, and restricting downstream genetic analysis. The amount of DNA present in the blastocoel varies considerably and appears to be undetectable in some embryos (or in a state which leaves it refractory to amplification). It is important to understand the basis of the variation in the results obtained from different studies. Is this variability related to differences in physiological processes between samples, and/or in embryo morphology? If so, the quantity and quality of BF-DNA may differ between the embryo cohorts used in studies; some focusing on potentially viable embryos, surplus from treatment, others concentrating on lower quality embryos deemed non-viable.

Secondly, it is critical to identify any subtleties of the BF extraction method which are critical to the success of amplification and analysis. The reported volume of BF samples

analyzed has ranged from 0.3nl (which probably represents a pure BF sample) up to 1µl (which must surely include additional fluid derived from the media or other sources). Differences in volume will clearly impact the concentration of BF-DNA and may have ramifications for subsequent amplification efficiency (Gianaroli *et al.*, 2014; Magli *et al.*, 2016; Palini *et al.*, 2013, Tober *et al.*, 2014; Tšuiiko *et al.*, 2018). Moreover, Magli *et al.* (2016) reported an increase in the detection of DNA in BF samples following WGA throughout the duration of their study, beginning at 50% at the start of their investigation and reaching 100% during the final year of their study. This suggests that the process of blastocentesis is associated with a significant learning curve. The authors speculate that the step of “tubing” the BF is a critical determinant of success, and note that optimal results were obtained following direct pipetting of the BF into cold PCR tubes after aspiration, followed by immediate centrifugation.

The principal cause of failed BF-DNA detection likely stems from its degraded nature. Degradation of primer annealing sites and DNA strand breaks in the sequences lying between primers could hinder PCR-based amplification strategies. The inevitable shortening of fragments caused by DNA degradation will be inhibitory to most WGA methods, especially those based upon MDA, which requires long intact DNA fragments for optimal amplification. The diagnostic efficiency of blastocentesis-based genetic analysis may ultimately require new WGA techniques, specifically designed for the efficient amplification of short DNA fragments. Such methods should ideally preserve the length of the DNA template in the sample and not cause further shortening of already degraded material.

In summary, procedural disparities, especially differences in BF collection storage, as well as variation in DNA amplification techniques and the quality of the embryos tested, are likely to have had a significant impact on the results obtained using blastocentesis. An optimal methodology for sampling, amplification and analysis of BF-DNA is yet to be determined, highlighting the importance of sharing and eventually standardising the protocols used. The

ideal embryological stage for BF sampling, the relevance of blastocyst morphology and the origin of BF-DNA will also require clarification if this methodology is to receive an adequate validation and, ultimately, wider clinical application.

## **Spent embryo culture medium**

A potential alternative for the non-invasive genetic assessment of preimplantation embryos involves the analysis of spent embryo culture media (SCM) (Figure 1). The past decade has seen several publications exploring the wide variety of components secreted into the medium by embryos (the secretome). Studies have focused on metabolites, proteins, interleukins and micro-RNAs and have considered whether measurement of certain secreted molecules may have value for the prediction of embryonic reproductive competence (Capalbo *et al.*, 2016; Huang *et al.*, 2017; Katz-Jaffe *et al.*, 2009; Sánchez-Ribas *et al.*, 2018). In terms of genetic analysis, there is evidence that SCM could harbor 6-7 times more DNA than found in the BF. This raises the possibility that SCM could provide a source of material for a completely non-invasive means of genetic testing, in contrast to blastocentesis which still requires the embryo to be subjected to a degree of micromanipulation (Galluzzi *et al.*, 2015).

**Figure 1: Methods of obtaining embryonic DNA during preimplantation genetic testing.**  
A) Traditional invasive methods comprise biopsy of both polar bodies, blastomere(s) from cleavage stage embryos, or trophectoderm cells from blastocyst stage embryos.  
B) Novel minimally invasive approaches of obtaining embryonic genetic material for analysis include analysis of DNA in spent embryo culture media and the blastocoel fluid of blastocyst embryos.

The detection of genomic DNA (gDNA) and mitochondrial DNA (mtDNA) in SCM have been reported as early as day-2-3 of development (Assou *et al.*, 2014; Galluzzi *et al.*, 2015; Hammond *et al.*, 2017; Stigliani *et al.*, 2013, 2014; Yang *et al.*, 2017). While certain

media formulations may contain DNA contaminants, a cause for concern when considering genetic testing, it has been shown that the amount of DNA increases during embryo culture, suggesting that much of the genetic material detected in SCM has an embryonic origin (Hammond *et al.*, 2017; Vera-Rodriguez *et al.*, 2018).

Although the preimplantation embryo is surrounded by a protective glycoprotein membrane (the zona pellucida), this has a high degree of permeability, even to relatively large macromolecules, and thus it is likely that nucleic acids can pass from the embryo into the medium with little resistance (Gwatkin, 1967; Hastings *et al.*, 1972; Legge, 1995). However, the mechanisms underlying release of embryonic DNA remain unclear. The reported PCR amplification rates achieved from SCM are inferior to those using cellular biopsy, indicating that as in the blastocoel, the gDNA in the SCM is likely of very low abundance and/or degraded and may be a product of cell death processes (Capalbo *et al.*, 2013; Galluzzi *et al.*, 2015).

Whilst apoptosis and associated DNA release appears a normal facet of blastocyst development, different processes may be needed in order to explain the presence of embryonic DNA in SCM at earlier stages. Studies have reported that healthy, normally developing human embryos display no hallmarks of apoptosis before the blastocyst stage and that the occurrence of cell death prior to compaction is associated with fragmentation and embryo arrest (Chi *et al.*, 2011; Hardy, 1999; Jurisicova *et al.*, 2003; Yang *et al.*, 1998). Accordingly, Stigliani *et al.* (2013) sought to investigate the significance of gDNA and mtDNA in day-2-3 SCM as a biomarker of embryo quality. Interestingly, total double-stranded DNA (dsDNA) concentrations were higher in SCM samples obtained from embryos of poor-to-average morphological grades compared to samples associated with high-quality embryos. Additional qPCR for single-copy nuclear and mitochondrial genes revealed that the dsDNA was mostly of a mitochondrial origin, and that mtDNA copy number in the medium was positively associated with both the degree of embryonic fragmentation and with advancing maternal age.

On the basis of these results, the authors concluded that the DNA in the SCM is derived predominantly from anuclear cytoplasmic fragments rather than apoptotic bodies. However, these data must be interpreted cautiously due to the use of a single-copy nuclear gene for normalization, which is suboptimal for the quantification of mtDNA analysis in samples containing very low amounts of DNA, especially if the DNA is degraded (Wells, 2017). Indeed, upon adopting both single- and multi-copy gene qPCR markers, Hammond *et al.*, (2017) detected mtDNA and gDNA in SCM from cleavage stage embryos displaying low levels or no fragmentation. Moreover, Stigliani's team (2014) later reported that the ratio of mtDNA to gDNA in day-3 SCM was positively associated with subsequent blastulation rates, trophectoderm quality and implantation outcomes. Currently, the extent to which embryo viability is indicated by the total level of gDNA, mtDNA, or the relative ratio of these types of genetic material in the SCM, remains to be confirmed.

#### **PGT-M using spent culture media**

Regardless of the mechanisms underlying the release of DNA into the medium, the key question for PGT is whether or not the material reflects the genetics of the embryo. In regard to PGT for monogenic disorders, the potential utility of DNA from SCM to detect male embryos, of potential value for the avoidance of X-linked disorders, has been evaluated in several investigations (Assou *et al.*, 2014; Galluzzi *et al.*, 2015; Yang *et al.* 2017). A promising study performed by Galluzzi *et al.* (2015) attempted to identify the sex of embryos by performing qPCR for Y-chromosome (*TSPY1*) and autosomal (*TBC1D3*) DNA sequences. After employing a sequential media system, *TBC1D3* amplified in 94.4% of 54 SCM samples collected following day-3-5/6 culture, whereas *TSPY1* amplified in only 41.2% of samples, indicating that this subset of embryos were male. Whilst this suggests that the sex could be characterized using SCM, the sex was only subsequently verified in five cases, by testing pregnancies resulting after the transfer of the respective embryos. Interestingly, of 32 additional



media samples collected from earlier day-1-3 culture, a comparable 93.7% exhibited successful *TBCID3* amplification whereas only 6.7% were associated with successful *TSPY1* amplification. The implication is that analysis following day-3-5/6 culture may confer a more robust sex determination when using this strategy, presumably due to higher DNA concentrations of embryo DNA in the media. However, it is noteworthy that another study demonstrated detectable *TSPY1* and *TBCID3* amplification in media controls that were unexposed to embryos during culture, highlighting the necessity of fully evaluating the presence of DNA contamination and concordance with the embryo when validating non-invasive PGT protocols (Hammond *et al.* 2017).

Some success has also been achieved in the detection of specific mutations responsible for inherited conditions using SCM (Table 3). Wu *et al.* (2015) demonstrated that the carrier status for a deletion mutation causing alpha-thalassemia could be determined from SCM using a nested-qPCR methodology. The carrier status of 413 embryos from 38 carrier couples was determined by analyzing day-3 blastomere biopsies using an alternative routine PGT-M method. Embryos with an unaffected genotype were used clinically following culture to day-5, while the remaining 202 embryos (affected or undiagnosed) were cultured in fresh media until day-6. Analysis of the day-5-6 SCM using the nested qPCR methodology achieved a result in 88.6% of samples. These data indicate that when analyzed using appropriate methods, DNA in SCM may provide a template suitable for amplification and analysis. However, the stress inflicted to the embryo during initial blastomere biopsy may have increased the quantity of DNA available in SCM for analysis, and we cannot be sure that similarly promising results would be obtained if the embryos were unmanipulated. Moreover, the concordance between paired samples were not reported, and thus the diagnoses remains to be demonstrated and the results can only be considered suggestive of clinical utility.

As an alternative means of analysis, some studies have applied WGA methods to SCM; reported amplification rates varying from 81.8-100% samples, generally higher than the rates achieved using BF samples (Feichtinger *et al.* 2017; Lane *et al.* 2017; Liu *et al.*, 2017; Shamonki *et al.* 2016; Vera-Rodriguez *et al.* 2018; Xu *et al.* 2016). These data, along with the observation that DNA in SCM can be amplified using an MDA-based WGA (which ideally requires template DNA fragments to be at least 2kb in length), suggest that the integrity and/or quantity of the DNA is superior in comparison with that found in the blastocoel (Shamonki *et al.*, 2016). In addition, SCM-derived WGA products sequenced at a depth of 30x reads reportedly confer up to 65% genome coverage - only slightly lower than the ~72% coverage obtained following genome sequencing of single diploid human cells (Huang *et al.*, 2015; Xu *et al.*, 2016). Whilst less important for PGT-A, which often utilizes low-pass genome sequencing approaches that require less than 0.1% of the genome to be sequenced, the extent to which the genome is represented in amplified samples is an important consideration for PGT-M, in which sequence data must be reliably obtained from specific genes/regions.

Using the WGA-PCR methodologies previously described for blastocentesis samples, Galluzzi *et al.* endeavoured to genotype the *MTHFR* C677T polymorphism in eight SCM samples collected following day-3-5/6 culture. Amplification and genotyping were successful in 62.5% of samples - rates that were higher than achieved using BF (44.4%), albeit still unacceptably low for clinical application. Only two paired trophectoderm biopsies were available; revealing concordance in one sample and incidence of ADO in the other. It is impossible to draw any firm conclusions from such small sample sizes, but it appears that while superior amplification might be achieved using SCM compared to BF, the ability to consistently obtain an accurate diagnosis might still be compromised.

To minimize diagnostic complications associated with ADO and suboptimal PCR amplification, it is typical for PGT-M strategies to supplement direct mutation detection in

biopsied cells with genotyping of multiple linked polymorphisms. This approach allows inheritance of the parental chromosomes carrying mutations to be tracked and the genetic status of embryos to be inferred. The first to adopt this strategy for SCM analysis was Liu *et al.* (2017), who sought to perform PGT-M for a beta-thalassemia causing mutation. Following culture, day-1-5 media was collected from two blastocyst stage embryos and eight fragmented or arrested embryos, and the embryos underwent a biopsy. For the samples in which MALBAC-WGA (Multiple Annealing and Looping Based Amplification Cycles, Yikon Genomics) was successful (80% of SCM and 100% of embryo biopsies), PCR was used to further amplify the hemoglobin beta subunit gene (*HBB*) and 10 SNPs in close proximity to the gene. Allele dropout was found to be frequent, with multiple loci affected, and the detection of anomalous SNP alleles raised concern over genetic contamination. Nevertheless, the seven SCM samples in which mutations were successfully genotyped using NGS displayed a diagnosis consistent with the corresponding embryo biopsy. The results of linkage analyses were also concordant and confirmed that the DNA was primarily of embryonic origin.

Similar observations were reported by Capalbo *et al.* (2018), who applied a qPCR-based PGT-M methodology to 72 SCM samples. Amplification occurred in 89.7% of the 378 loci tested in SCM; rates inferior to that achieved using trophectoderm biopsies (100%), but much higher than the same team had achieved using BF samples (27.4%). Regardless, there was evidence to indicate that the accuracy of the diagnosis was compromised by DNA contamination of maternal origin. Only 20.8% of SCM samples displayed full haplotype concordance with paired trophectoderm biopsies and ADO rates were higher for alleles of paternal origin compared to those of maternal origin (14.2% vs 8.2%). This suggests that an excess of maternal DNA, presumably from contaminating sources such as cumulus cells, might be present. In addition, testing of four (of 12) trophectoderm samples indicated the

corresponding embryos to be free from the mutation, yet the SCM samples for these embryos each displayed detectable levels of the maternal mutant allele.

In conclusion, whilst DNA in the SCM appears to be of superior integrity and greater quantity compared with that found in the blastocoel the amplification rates are still lower, and ADO rates higher, than observed using conventional trophectoderm biopsy. As with standard PGT-M, linkage analysis approaches may help to mitigate these technical hindrances, however, the potential presence of genetic contamination in SCM is a significant concern. The clinical consequences of this could be severe; potentially leading to misdiagnosis of embryos and the birth of affected offspring.

#### **PGT-A using spent culture media**

The potential for SCM to provide a source of DNA for PGT-A analysis has ignited huge interest in reproductive genetics laboratories worldwide. Whilst different studies have reported variable success rates, analyses of SCM following WGA and NGS have generally succeeded in yielding chromosome copy number information for the majority of samples tested. However, as with BF analysis, concordance rates between SCM and whole embryos/biopsy specimens have varied considerably between studies (Table 4).

One of the first investigations attempting to acquire ploidy data from SCM examined day-3-day-5/6 media from embryos undergoing sequential culture, amplifying the DNA with MDA (REPLI-g, Qiagen) and assessing chromosomal status using aCGH (Shamonki *et al.*, 2016). Whilst detectable levels of DNA were obtained for 96.5% of 57 samples, quality control metrics suggested that the accuracy of chromosome copy number evaluation was likely to be suboptimal in the majority of samples. This may reflect insufficient quantity or integrity of DNA in SCM, although the poor data quality may also relate to a lack of sensitivity of molecular methods that have not been optimized specifically for the purpose of analyzing DNA

in SCM. It is noteworthy that superior cytogenetic data was produced in two other studies adopting the use of MALBAC for WGA (Yikon Genomics) and an NGS-based methodology for chromosome copy number assessment (Liu *et al.*, 2017; Xu *et al.*, 2016).

In an analysis of day-1-5 SCM obtained following culture of 88 embryos from seven couples, Liu and colleagues achieved successful WGA in 90.9% of samples - rates identical to those achieved using counterpart trophectoderm biopsies. It should be noted, however, that a 90% amplification rate for TE specimens is significantly lower than observed for this sample type in most published studies. Although the amplification of DNA was observed in the majority of SCM samples, the mean DNA concentration was lower than that typically obtained from TE samples (26.2ng/μl vs 60.2ng/μl). Nevertheless, the authors considered the amplified DNA to be of sufficient quantity and quality to obtain chromosome copy number information in 56 paired media and biopsy samples. Unfortunately, the rates of full chromosome concordance between the samples (including >40% mosaicism and segmental aneuploidies) were relatively low: 64.5% when SCM samples were compared with 31 blastocysts; 44.0% when compared with the 25 arrested or degenerated embryos. Interestingly, rates of agreement were higher for blastocysts that had been defined as euploid (according to the analysis of a TE biopsy) (n=20, 90.0% concordance). This suggests that concordance may be influenced by factors such as embryo quality and viability. In addition, the concordance rate was 83.9% when mosaicism, whole chromosome and segmental aneuploidies were grouped, and blastocysts were given an overall classification of chromosomally 'normal' (euploid) or 'abnormal'. These observations may give an insight into how best to interpret SCM data in order to understand the embryos genetic status during NI-PGT-A.

The ability of MALBAC-NGS methodologies to consistently obtain a PGT-A result using SCM was also demonstrated by Xu *et al.*, who evaluated ploidy concordance between day-3-5 SCM and counterpart whole blastocysts, using 42 warmed vitrified cleavage stage

embryos from 17 couples. Encouragingly, a chromosome copy number evaluation was successfully obtained in 100% of samples. This represents the highest rate achieved by any study to date. However, it is possible that the use of vitrification and thorough washing of embryos might have resulted in small amounts of damage and a higher rate of cell death, increasing the likelihood of embryonic DNA being released into the SCM. Similar to the study of Liu and colleagues, 36 paired samples (85.7%) displayed concordance with respect to whether they were defined as euploid or chromosomally abnormal (including mosaicism, whole and segmental aneuploidies). The assay also identified a chromosomal imbalance in SCM samples when the corresponding embryos were mosaic (n=4). The authors speculated that false positives in detecting abnormalities from SCM samples (4 of 25) stemmed from mosaicism or the preferential elimination of aneuploid cells, whilst false negatives (2 of 17) were a consequence of genetic contamination, although no genotyping data was provided to confirm this.

Although a significant level of disagreement remained between results from SCM and paired blastocysts, and the rates of full chromosome concordance were not determined, Xu *et al.* proceeded to use their 'noninvasive chromosome screening' (NICS) assay clinically during IVF for seven couples with balanced translocations, azoospermia or recurrent pregnancy loss. For each patient, only embryos diagnosed as chromosomally normal by NICS analysis of their paired day-3-5 SCM samples were considered for single elective transfer. Following up to two transfer cycles, six couples achieved successful pregnancies, whilst only one experienced implantation failure. It is noteworthy that an additional biopsy was not performed for these embryos and that the decision of whether to transfer an embryo was based purely on the NICS assay result, which is risky provided the inconsistent evidence currently backing SCM analysis. Moreover, no control group was used in this study. Regardless, five of the pregnancies had resulted in the delivery of chromosomally normal offspring at the time of publication. In

addition, a clinical trial is currently ongoing in order to further validate the accuracy of the chromosomal status results obtained from spent medium using the NICS assay, against that obtained from the paired embryo using conventional invasive PGT-A methodologies (ClinicalTrials.gov ID: NCT03879265).

Adopting a different approach to analysis, Feichtinger *et al.*, (2017) evaluated the ploidy agreement between 22 PB biopsies and paired day-1-5 SCM samples, following continuous culture of healthy and arrested embryos obtained from couples of advanced maternal age. The WGA strategy adopted (SurePlex, Illumina) successfully amplified 81.8% of media samples – rates lower than achieved using PB samples (100%) and also poorer than previous reports using MALBAC-based amplification for SCM. Importantly, however, the hampered amplification rates observed in this study may relate to the use of only 5µl media for analysis, out of the 25µl of media used for embryo culture, rather than the WGA technology adopted. Regardless, subsequent aCGH-based PGT-A obtained a ploidy result in all amplified samples, revealing that media samples frequently displayed the embryonic aneuploidy predicted from the corresponding PB result. Overall, there was a 72.2% agreement in terms of ploidy status and 48.6% concordance for single chromosomal aneuploidies between matched samples. Whilst these concordance rates were somewhat lower than reported by Xu and colleagues comparing SCM and TE biopsies, they are similar to concordance levels previously reported for PBs and matched trophoctoderm biopsies (61.7% and 38.1%, respectively) (Capalbo *et al.*, 2013).

To investigate the underlying causes of discrepancies between SCM and PB results, the researchers subsequently analyzed six media samples that had not been exposed to oocytes or embryos and also spent media following culture of four denuded oocytes. Amplification only occurred in the latter portion samples, suggesting that the media itself was free of DNA, but revealing the presence of genetic material associated with the oocytes. The oocytes are unlikely

to have lost their own DNA into the media and consequently this material is likely to represent a form of contamination, possibly originating from cumulus cells or other procedure-related sources. It is reasonable to speculate that the slightly higher rates of concordance achieved in some studies that have examined day-3-5 culture media, as opposed to studies looking at SCM associated with earlier stages or continuous media, may have benefitted from a reduction in the levels of contaminating DNA. Extended culture provides more time for DNA derived from sperm and cumulus cells to degrade and a change of media on day-3 should eliminate, or at least dilute, any remaining contaminants. It also seems likely that more advanced embryos, composed of larger numbers of cells, might be associated with greater quantities of DNA in the SCM, perhaps reducing the relative contribution of any contaminants.

Improved results when examining SCM samples from later stages of preimplantation development were also reported by Lane *et al.* (2017), who performed NGS-based PGT-A on 178 paired SCM and clinical biopsy samples following 24 to 48h incubation in fresh media commencing either day-3, 4 or 5. An initial WGA step using PicoPlex or DOPlify (RHS) kits achieved favorable amplification rates of 94% in SCM samples. Day-3-5 SCM and paired biopsy specimens showed low rates of concordance; 21.4% of male embryos were misdiagnosed as female, and autosome concordance rates were only 65.4%, with the majority of cases of discordance a consequence of false negative aneuploidies in the SCM. Interestingly, however, when SCM was analysed from embryos which had undergone a later media change at day-4, embryo ploidy rates rose to >95% and sex chromosome concordance rates were 100%.

The magnitude of the problem that maternal contamination poses for accurate PGT-A analysis using SCM was further demonstrated by Vera-Rodriguez *et al.* (2018), in one of the most comprehensive studies on the topic to date. Following sequential culture of 60 embryos, day-3-5 SCM samples were subjected to a double WGA amplification due to the expected low



DNA concentration; an initial SurePlex (Illumina) amplification was followed by a second round using the Ion ReproSeq PGS kit (ThermoFisher Scientific). In contrast, paired trophoctoderm biopsies were amplified using only the Ion ReproSeq PGS kit. It should be noted that a double WGA strategy would require extensive validation, as the risk of amplification biases occurring is likely to be high, potentially leading to errors, especially when trying to detect small copy number variations (Deleye *et al.*, 2015). Using this approach, chromosome copy number information was determined using NGS in 91.6% of SCM samples, while 100% of trophoctoderm biopsies yielded a result. Samples which were assigned a euploid female karyotype (46, XX) following trophoctoderm analysis were excluded from the assessment of concordance, due to the inability to differentiate from maternal contamination. Of the remaining 51 samples, only 33.3% of paired samples were concordant in their aneuploidy diagnosis (the lowest concordance rate reported amongst studies), whilst the remaining samples displayed chromosomal patterns consistent with maternal contamination.

To confirm the origin of DNA in SCM more precisely, paired follicular fluid and trophoctoderm biopsies were sequenced to identify 124 informative SNPs by which maternal and embryonic haplotypes could be distinguished (Vera-Rodriguez *et al.*, 2018). This approach revealed maternal DNA in all media samples tested (n=35), including those where contamination had not previously been detected using NGS. This provides evidence that contamination may be the predominant cause of diagnostic discord between SCM and embryo biopsy specimens. However, it is worth remembering that targeted sequencing of individual polymorphisms provides an extremely sensitive genotyping method and it is possible that some of the detected contaminants may be of too low concentration to seriously impact cytogenetic analysis. Importantly, contamination was not the only caveat to accurate diagnosis; fluorescence *in situ* hybridization (FISH) analysis in a subset of whole blastocysts available (n=12) revealed mosaicism in 91.7% of samples. While some of these instances of mosaicism

may be artefacts, due to the inherent limitations in the accuracy of FISH, an appreciable level of mosaicism in the tested embryos seems likely, and thus may contribute to the discrepancy between SCM and TE biopsy results. All of the whole blastocysts analysed by FISH had previously produced a PGT-A diagnosis which was discordant between their paired SCM and trophectoderm biopsies, and interestingly, 75% of the chromosomes analyzed in the whole blastocysts were in agreement with the diagnosis derived from analysis of trophectoderm DNA rather than SCM. This perhaps represents evidence for an aneuploidy-correction mechanism possessed by embryos, as discussed in more detail below.

In conclusion, studies assessing the potential of non-invasive PGT using SCM have shown potential in fulfilling the clinical requirement of being able to consistently obtain a diagnosis. However, in the majority of cases, rates of concordance are relatively low and would likely be considered unacceptable for most clinical applications. Current literature indicates that discordance of cytogenetic results from SCM with respect to embryo biopsy samples, may stem from embryonic mosaicism, the preferential elimination of aneuploid cells and/or DNA contamination, predominantly of maternal origin.

## **The origin of extra-embryonic DNA**

In order to fully evaluate the extent to which DNA in the BF and SCM represents the developing embryo, it is crucial that its origin is determined. It is thought that a proportion of extra-embryonic DNA may be released as a consequence of processes of cell death in the embryo, such as apoptosis or necrosis - both of which are associated with DNA fragmentation (Zhivotosky and Orrenius, 2001). Apoptosis occurs in healthy human blastocysts with hypothesized roles in the regulation of cell number, or the elimination of cells which are genetically abnormal or otherwise developmentally compromised (Hardy, 1997, 1999; Hardy

*et al.*, 1989). Apoptotic cells may be either phagocytosed by neighboring cells or expelled into the perivitelline space or blastocoel cavity (Fabian *et al.*, 2005). A characteristic of apoptosis is endonuclease cleavage of exposed and accessible internucleosomal linker DNA, producing oligomers of around 180bp and multiples of this size (Matassov *et al.*, 2004). Consistent with the possibility that extra-embryonic DNA originates from apoptotic cells, NGS analysis of pooled BF samples (n=3) revealed two native populations of DNA fragments; the first 160-220bp in length, and the second spanning 300-400bp (Zhang, Li, *et al.*, 2016). A wave of apoptosis has been described in the ICM of many mammalian species, although in human IVF embryos levels in both ICM and TE lineages appear to be similar (7-8% of cells) (Brison, 2000; Hardy *et al.*, 1989). Whether these discrepancies are due to differences between species or are a consequence of the manipulations involved in IVF remain unclear. If apoptosis is largely constrained to the ICM, it is conceivable that extra-embryonic DNA could provide more relevant information concerning the lineage that will ultimately produce the fetus than conventional TE biopsy.

Through tracking of individual cells in a chimeric preimplantation mouse model of mosaicism, Bolton *et al.*, (2016) observed that aneuploid cells in the murine TE persist with proliferative abnormalities, whilst those in the ICM are removed by apoptosis. If DNA is preferentially released from aneuploid cells, perhaps as part of a process of euploidization in mosaic embryos, there could be increased risks for false positive diagnosis of aneuploidy – the DNA ejected into the BF being aneuploid while the cells remaining in the ICM are euploid. It is known that some mosaic embryos can implant and produce euploid births, suggesting that mechanisms for the elimination of aneuploid cells may exist (Fragouli *et al.*, 2017; Greco *et al.*, 2015; Lledó; *et al.*, 2017). Hopefully, an insight into the origin of extra-embryonic DNA should be obtained from an ongoing multicenter study, in which an additional ICM biopsy is undergoing PGT-A analysis, in addition to SCM samples and a paired TE biopsy

(ClinicalTrials.gov ID: NCT03520933).

Relevant to this idea, a study by Lagalla and colleagues demonstrated that embryos that underwent abnormal cleavage divisions, of a type known to produce aneuploidy and chromosomal mosaicism, were sometimes able to form euploid blastocysts. These chromosomally normal blastocysts typically displayed exclusion of some cells upon compaction, and it was shown that while biopsies taken from these developing embryos were euploid, the excluded cells were aneuploid. The possibility of self-correction of mosaic embryos via apoptotic or other processes of cell loss/exclusion warrants further investigation in human embryos. The limited information available from published studies on extra-embryonic DNA suggests that there may indeed be a higher number of aneuploid events in BF and SCM samples in comparison with paired TE biopsies (Magli *et al.*, 2018; Tšuiiko *et al.*, 2018). If confirmed, this could call into question the premise upon which analysis of extra-embryonic DNA for PGT-A is based.

It is important to understand whether DNA detected in the BF and SCM is released as a consequence of physiological or pathophysiological processes. Is more DNA available in the BF/SCM of embryos of good morphology, which have many cells, or in those of poor viability and morphology, which might have increased rates of cell death? Thus far, studies have yielded conflicting results in regard to the association between successful retrieval of extra-embryonic DNA and embryo quality. Whilst Zhang, Li, *et al.*, reported no association between embryo quality and quantity of BF-DNA, other groups have observed that WGA efficiency was positively correlated with blastocyst morphology and that fully expanded day-5 blastocysts were the more likely to yield detectable extra-embryonic DNA than less developed embryos (Capalbo *et al.*, 2018; Magli *et al.*, 2016). Rule *et al.* (2018) reported positive associations between both embryo quality and BF-DNA content, and between BF-DNA content and caspase-3 activity – a marker of apoptosis. Conversely, the study by Magli

*et al.* (2018) reported that WGA rates (SurePlex, Illumina) were significantly higher in a cohort of embryos with an aneuploid trophectoderm biopsy diagnosis (81% of 185 samples) than those with a euploid diagnosis (45% of 71 samples). Moreover, when 53 paired embryos were used for IVF, clinical pregnancy rates were higher in the group with failed BF amplification, compared to those with successful amplification (77% vs 37%). Based upon these results, the authors speculated that failed BF amplification might be able to serve as an additional selection criterion to prioritize embryos for transfer during IVF.

It also remains a possibility that at least a portion of the DNA in the blastocoel is a product of damage to the embryo, caused by the sampling procedures themselves. Penetration of the TE layer during blastocentesis could result in the accidental lysis of cells, releasing their nuclear contents into the BF, which is subsequently aspirated. It is also unclear whether extra-embryonic DNA exists in a cell-free state or whether it is sometimes membrane-enclosed, contained within cells excluded from the embryo. The diameter of the ICSI pipette typically used for collection of BF is such that sampling of whole cells floating in the BF cannot be entirely excluded. The discovery of micro-RNAs (miRNAs), extracellular vesicles and proteins in human BF indicates that the blastocoel may have an active function in facilitating intercellular communication during embryo development and implantation (Battaglia *et al.*, 2019; Poli *et al.*, 2015). It is possible that a portion of extra-embryonic DNA may also exist within such extracellular vesicles.

### **Genetic contamination**

Any procedure that involves the amplification of a small quantity of DNA carries a risk of contamination. Intuitively, the analysis of culture media may carry greater risks than blastocentesis, since the fluid being tested exists in a comparatively open system.

Concerningly, Vera-Rodriguez *et al.* (2018) estimated the median percentage of embryonic DNA in SCM to be only 8%, the rest presumably being non-embryonic contaminants. Contamination may stem from 4 major origins: 1) it may enter the medium (or individual media components) during the manufacturing processes; 2) it could be maternally-derived from cumulus cells or polar bodies; 3) it could be paternal, originating from sperm cells; 4) or it could arise from exogenous sources such as from laboratory personnel. Low levels of contaminating DNA were also recently reported in commercial media commonly used in NI-PGT studies (Hammond *et al.*, 2017). Further analysis confirmed supplementary human serum albumin (HSA) as a predominant source of contamination. HSA is frequently added to media as it contains a variety of components thought to be beneficial for embryo viability and development (Meintjes *et al.*, 2009; Otsuki *et al.*, 2013). However, with a high affinity for DNA binding, HSA supplementation may enhance susceptibility to contamination (Malonga *et al.*, 2006).

To date, the majority of studies investigating PGT using SCM have not controlled for all potential sources of contamination, thus failing to confirm that their results are based purely on embryonic DNA. To minimize the likelihood and impact of contamination, it is advisable for oocytes to be fully denuded of cumulus cells and it seems reasonable to recommend the use of ICSI for fertilization. In cases where IVF is used for fertilization, it is conceivable that sperm-derived DNA might be present in the SCM, although its highly condensed nature reduces the risk that it will be inadvertently amplified. Moreover, fresh media, unexposed to embryos, should be incubated alongside the embryo culture drops, in identical conditions, and be subjected to testing in parallel with SCM samples in order to control for contamination.

It is also critical that new protocols for the analysis of SCM samples are developed, minimizing the risk of contamination and/or allowing embryonic DNA to be distinguished from potential contaminants. Various strategies could also be envisaged that might permit

analysis of a DNA population exclusively of embryonic origin. For example, if there are differences in the length of DNA fragments derived from embryos compared to those originating from contaminants, it may be possible to focus analysis on one of the two populations. This would be analogous to NGS protocols used for non-invasive prenatal testing (NIPT), which are able to determine the relative fractions of the cell-free DNA found in the maternal circulation attributable to the fetus and to the mother by assessing differences in DNA fragment length. In addition, if a portion of DNA in SCM were found to exist in exosomes, a targeted analysis of membrane bound vesicles may present a means of evaluating only embryonic DNA. Another possibility could involve undertaking NGS followed by analysis of individual alleles at heterozygous sites, revealing DNA fragments of embryonic origin by the presence of paternal-derived alleles and additionally, with careful measurement of the number of reads attributable individual maternal alleles, potentially detecting an embryonic pattern even when it is overlaid and diluted by a maternal genetic signature.

#### **Protocol optimization and standardization**

A further requirement if NI-PGT is to be used clinically involves the optimization and standardization of culture conditions and medium retrieval protocols. Initial studies indicate that DNA accumulates in the medium throughout preimplantation development, with the highest rates of amplification and diagnostic accuracy achieved following analysis of culture media samples from day-5-6 (Galluzzi *et al.*, 2015; Wu *et al.* 2015; Hammond *et al.* 2017). In clinical practice, embryos are cultured using sequential media systems or continuous single media formulations. The latter may be either entirely uninterrupted or replenished on day-3. DNA has been reported in SCM as early as day-2, although presumably there is little if any representation of the nuclear genome from viable embryos at such early stages (most of the genetic material present is probably mitochondrial, derived from cellular fragments). As

discussed above, a change or renewal of medium on day-3 may help to eliminate contaminants, but it will also mean that DNA entering the medium during the first three days of culture is likely to be lost.

The ideal time for sample collection, the stage of development and the optimal length of media exposure to the embryo will all need to be determined. It would also be of value to investigate whether different commercial brands of medium impact the quantity and integrity of the DNA released into by the embryo, as well as determining whether any DNA contamination is present. Embryos cultured in imperfect conditions display higher rates of cell death (Devreker and Hardy, 1997; De Hertogh *et al.*, 1991) and thus the detection of elevated quantities of DNA in the medium may also serve as an indicator that culture is suboptimal and requires improvement.

Studies investigating NI-PGT have contained many independent variables, which may influence the differences in diagnostic efficiency observed. In particular, the volume of SCM collected for amplification has varied greatly and studies have differed in whether the total volume of spent media or only an aliquot was used for subsequent amplification and testing (Table 5). This potentially impacts the proportion of the embryonic DNA which is collected and alters the initial template concentration. Determining the optimal volumes for the culture and analysis of embryos using NI-PGT is challenging. From observations made in our own laboratory, media components can have an inhibitory effect on DNA amplification during WGA and PCR, making it difficult to analyze SCM samples of volumes routinely used for embryo culture. Moreover, most commercially available WGA methods are designed for use with DNA/cell samples contained in very small volumes (generally <10µl). If samples of larger volume are used, reaction components must be scaled up, increasing costs and necessitating additional optimization.

One means of circumventing these technical issues would be to perform culture in



smaller volumes of media, thus avoiding excessive dilution of any DNA released by the embryo and minimizing the quantity of inhibitory media components. A single study reported that embryo culture performed in lower volumes (7 $\mu$ l vs 35 $\mu$ l) improved day-5 blastocyst formation rates, perhaps a consequence of concentration of autocrine factors produced by the embryo (Minasi *et al.*, 2015). However, commercial media have been validated by their manufacturers under specific conditions, and deviation from the recommended protocols, such as reduced culture volume, would require validation to ensure that embryo development and viability is not negatively impacted. Additionally, the handling of small droplets of medium is more difficult and the increased surface area to volume ratio leads to rapid evaporation (prior to oil overlay) and faster absorption of gases, which in turn affect osmolality and pH. It may also be problematic to employ lower volumes of culture media in laboratories using standardized time-lapse systems for embryo culture.

Given the issues and uncertainties related to embryo culture in small volumes, the development of alternative means of concentrating the DNA in SCM for analysis remains desirable. Although multiple strategies exist for the concentration of DNA (e.g. using a vacuum for evaporation), these often concentrate the PCR-inhibitory salts in the media too. Other common methods, such as utilizing affinity columns/beads or precipitating DNA, result in some loss of material and are therefore unsuitable for working with the extremely low quantities of DNA found in SCM.

Studies have also differed in their sample handling procedures; especially in terms of whether vitrified-thawed or fresh embryos were used. This is significant as cryopreservation protocols, which inflict non-physiological chemical and physical pressures, may damage the DNA in cells that are destined to release their contents into the SCM, thereby reducing amplification efficiency when PCR methods are applied (Kopeika *et al.*, 2005; Park *et al.*, 2006; Valcarce *et al.*, 2013). Conversely, cryopreservation can also damage the cell membrane,

which may increase the likelihood of embryo cells releasing their contents into the SCM, and thus improve the template concentration available for amplification, but at some cost to the embryo (Kopeika *et al.*, 2015).

It would be useful to determine whether SCM could be collected daily and frozen in order to prevent the continued degradation of the extra-embryonic DNA it contains, with the accumulated samples finally pooled together and tested as a single entity. However, even if successful, such a strategy would be difficult to implement clinically, unless some sort of microfluidic system of media sampling could be developed. Lastly, studies differ in their use of assisted hatching and embryo biopsy. It remains unclear whether methods such as these, which disrupt the zona pellucida and risk damaging cells, affect the quantity of DNA which escapes into the medium.

Interestingly, three recent studies have considered whether PGT-A could be enhanced by combining information from both blastocoel fluid and SCM samples derived from a single embryo (Kuznyetsov *et al.*, 2018; Li *et al.*, 2018; Ben-Nagi *et al.*, 2019). In one study, involving actual clinical application, an attempt was made to collect independent cytogenetic data from SCM, blastocoel fluid and TE biopsy samples from embryos prior to uterine transfer. It was hoped that the consideration of genetic information from these three distinct sources might provide a more robust diagnosis (Ben-Nagi *et al.*, 2019). The other two studies combined DNA from the blastocoel and SCM, aiming to reduce the likelihood of encountering amplification failure by gathering as much extra-embryonic DNA as possible into a single sample. Kuznyetsov *et al.* (2018) cultured 28 cryopreserved blastocysts for 24h to facilitate re-expansion, before laser collapse of the embryo to allow the blastocoel contents to spill into the culture medium. The combined samples, called ‘Blastocoel Contents and Culture Media’ (BCCM), were subjected to WGA (SurePlex) and NGS-based chromosome copy number analysis (VeriSeq, Illumina). Paired TE biopsies taken prior to cryopreservation, and the

remaining whole embryo were available for analysis. Moreover, 19 additional fresh embryos underwent day-4-5/6 culture prior to TE biopsy, blastocyst collapse, and BCCM analysis.

Amplification was successful in 100% of 47 BCCM samples, indicating that this strategy may have advantages in consistently obtaining DNA for analysis. Samples derived from cryopreserved embryos displayed ploidy concordance rates of 87.5% and 96.4% with TE biopsy and the whole blastocyst (WB) samples, respectively – very similar to the 91.7% rate observed when TE samples were compared to the remaining blastocyst. In addition, whole chromosome copy number concordance rates were 99.3% (BCCM and TE), 99.7% (BCCM and whole blastocyst) and 99.7% (TE and whole blastocyst). These favorable results were replicated in BCCM samples derived from fresh embryos, which displayed ploidy agreement and whole chromosome copy number concordance rates of 100% and 98.2%, respectively, with respect to TE samples. Whilst these data present some of the highest rates yet achieved, when all aberrations including segmental aneuploidies and mosaicism were included, concordance rates dropped to 73.7% (BCCM and TE) for fresh samples, and 66.7% (BCCM and TE) and 78.5% (BCCM and WB) for frozen-thawed samples. It is worth noting, however, that a similar decline in concordance rate was observed between TE biopsy and WB samples when all abnormalities were considered (75%).

Similar amplification data were reported by Li *et al.* (2018), who cultured 40 embryos in sequential media before breaching the zona pellucida, collecting BCCM samples, then performing trophectoderm biopsy. All samples were subjected to MALBAC-WGA and NGS analysis, achieving successful amplification in 97.5% of BCCM samples and yielding an average concentration of 62.6ng/μl – similar to the average concentrations of 58.03ng/μl and 56.2ng/μl obtained after amplification of corresponding TE biopsies and remaining whole blastocysts respectively. The promising data reported by Li and Kuznyetsov represent some of the highest amplification rates to date, indicating that combining the BF with SCM may provide

an effective means of increasing the amount of embryonic DNA available. However, it is also possible that the use of frozen-thawed embryos, and laser collapse, in these studies may have inflicted accidental damage to cells of the embryo, thus potentially contributing to higher levels of DNA released into the medium.

Similar to the data reported by Kuznyetsov and colleagues, rates of full karyotype concordance (including segmental aneuploidies and mosaicism) between TE biopsies and the remaining embryo were 67.5%. However, BCCM samples displayed lower rates of concordance with counterpart embryos, of 47.5% (BCCM and WE) and 42.5% (BCCM and TE). Negative controls confirmed no baseline DNA contamination in media, and the sex chromosome diagnosis agreed between all three sources, indicating that maternal contamination was rare or absent. Moreover, the rates of mosaicism in samples (27.5%) was not considered to be higher than would be expected in a population of blastocysts. One major difference was the quality of samples used; the majority of embryos used by Kuznyetsov and colleagues were donated for research after being diagnosed as chromosomally abnormal, whilst Li and colleagues used embryos with a good morphological score. In addition, Kuzneytsov and colleagues used SCM obtained at a slightly later stage of preimplantation development than Li and colleagues. How these differences may influence the underlying discrepancies between these two studies remains unclear. Whilst the ability to consistently obtain an accurate diagnosis by combining the BF and SCM shows some promise, gaining an understanding of the extent to which DNA from each source independently reflects the genetics of the embryo is advisable.

#### **Analyzing concordance**

Discrepancies in the reported success rates between studies may result from differences in the processing, analysis and reporting of data. Results may be particularly impacted by the

1115 varying technologies and bioinformatic pipelines used to determine chromosome copy number.  
1116 Many studies on NI-PGT-A have adopted commercially available bioinformatic software for  
1117 cytogenetic analysis. These packages are optimized for, and validated using, cellular biopsies  
1118 rather than the degraded (and potentially cell-free) DNA found in the SCM and BF. It is  
1119 possible that the development of customized methods of DNA amplification and analysis,  
1120 tailored to extra-embryonic DNA, might help in obtaining a more accurate diagnosis.

1121 Challenges also arise in validating NI-PGT assays due to uncertainty over the true  
1122 karyotype of the embryos tested. Studies have typically considered concordance between the  
1123 BF/SCM and an embryo biopsy or the whole remaining embryo. Most studies have reported  
1124 concordance for overall ploidy status (euploid versus aneuploid), full chromosome  
1125 concordance (comparison of exact karyotypes) and/or concordance per single chromosome  
1126 (agreement for each chromosome examined individually and then summed together). High  
1127 levels of ploidy concordance are encouraging considering that this is the parameter upon which  
1128 clinical decisions regarding embryo transfer are based (i.e. euploid embryos may be  
1129 transferred, but those that are aneuploid are not). However, such analyses have accompanying  
1130 limitations – particularly considering that rates of aneuploidy are affected by maternal age. If  
1131 a particular analytical platform were prone to generating false positive or negative results using  
1132 BF/SCM, then the rates of ploidy concordance with the embryo would differ depending on the  
1133 maternal age of the population involved in the study (e.g. false positives will have less impact  
1134 on euploid/aneuploid concordance rate when the maternal age is higher, since most embryos  
1135 will be aneuploid anyway). Moreover, one must call into question the biological and/or  
1136 technical logic behind considering, for example, an aneuploid embryo with a TE biopsy  
1137 diagnosis of 47,XX,+5 to be concordant with an aneuploid BF or SCM karyotype of 46,XX,-  
1138 1,-3,+7,+9.

1139 Full chromosome concordance may present a more accurate measure of diagnostic

reliability; providing 24 distinct data points per sample instead of one. However, NI-PGT studies have differed in their definition of full concordance – particularly, in whether all chromosomal aberrations (e.g. segmental aneuploidies and mosaicism) were included in their analysis. Indeed, in some of the studies, it has not been clear whether the methods used were capable of detecting subchromosomal and mosaic abnormalities. Matters are further complicated by scenarios in which a whole chromosome gain/loss is seen in one sample and a mosaic abnormality affecting the same chromosome is seen in its counterpart, and cases of ‘reciprocal aneuploidy’ (e.g. a trisomy in the TE biopsy, accompanied by a monosomy for the same chromosome in the SCM or BF). The lack of standardization for data reporting creates difficulties in directly comparing the performance of different studies.

Another limitation of examining full chromosome concordance is that whilst an 100% accurate assay would, in theory, achieve total karyotypic concordance between NI-PGT-A and an embryo biopsy, in practice it is impossible to obtain ‘true data’ for an embryo due to the possibility of mosaicism. In other words, a discordant result does not necessarily mean that a technical error occurred, it may be the result of a genuine difference in the cytogenetic composition of different parts of the embryo. The ideal validation of NI-PGT assays would involve multiple biopsies of embryos donated for research, including the ICM, however, even this would not entirely overcome the issue of mosaicism. It is also possible that dilution of DNA from aneuploid cell lines, and controlled fragmentation, could be employed to mimic the low-concentration, degraded material found in BF and SCM, thus providing a sample where the ‘true’ cytogenetic status is known. However, such experimental simulations will only be feasible once the quantity and integrity of the nucleic acids in these sorts of sample are more fully characterized.

## **Conclusion**

The recent discovery of DNA in the BF and in SCM has generated a surge of interest in the potential for minimally-invasive PGT. There is little doubt that the elimination of embryo biopsy would convey economic and practical advantages for the genetic evaluation of embryos, as well as carrying little if any risk of damage to the embryo. However, the reliability of NI-PGT strategies, with respect to the proportion of samples yielding data and the concordance of genetic results relative to those obtained from whole embryos or biopsy specimens, has varied widely between published studies. The DNA found in BF and SCM is of relatively low abundance and poor integrity, presenting technical challenges for genetic analysis, which seem to be more successfully overcome by some strategies of sample collection and DNA amplification than others. It is not clear at present which laboratory methods are the most appropriate for the investigation of extra-embryonic DNA, although as more studies are published a consensus may begin to emerge. It would not be surprising if amplification methodologies that seek to preserve the length of the DNA fragments prove to be advantageous, since further shortening of DNA that is already somewhat degraded is likely to negatively impact downstream analysis, but this remains to be conclusively demonstrated. Uncertainty over the optimal method for NI-PGT, and questions concerning the reliability and clinical utility of the data produced, suggest that PGT based upon blastocentesis or SCM samples should, at present, only be carried out in the context of pre-clinical studies and carefully designed clinical pilot investigations. Crucially, for accurate data interpretation, and to avoid clinical misdiagnosis, the origin of extra-embryonic DNA must be confirmed and the causes underlying discordance with results from embryo biopsy specimens investigated. In particular, the possibility that aneuploid cells are preferentially eliminated from mosaic embryos via processes of apoptosis or expulsion of cells during compaction is highly relevant for clinical diagnosis.

It is clear that more work needs to be undertaken in order to define optimal methods for analysis of extra-embryonic DNA and to answer important biological questions concerning its origin. Nonetheless, the publication of promising results from some studies, reporting successful DNA amplification in a high proportion of samples and accurate assignment of karyotype and/or genomic sequences in the majority of cases, will inevitably continue to spur interest in the use of this material for genetic testing. Further studies should be encouraged and it will be fascinating to follow this evolving field of research and to see whether the clinical promise can ultimately be realized.

#### **Declaration of the authors' roles**

ML drafted and revised the manuscript.  
DW revised and edited the manuscript.

#### **Acknowledgements**

ML was supported by the University of Oxford Clarendon Fund and the Nuffield Department of Women's and Reproductive Health (NDWRH).  
DW was supported by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre Programme.  
Both authors have no conflict of interest to declare.

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