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REVIEW ARTICLE

An Update on Non-invasive Approaches for Genetic Testing of the Preimplantation Embryo

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Abstract: Preimplantation Genetic Testing (PGT) aims to reduce the chance of an affected pregnancy or improve success in an assisted reproduction cycle. Since the first established pregnancies in 1990, methodological approaches have greatly evolved, combined with significant advances in the embryological laboratory. The application of preimplantation testing has expanded, while the accuracy and reliability of monogenic and chromosomal analysis have improved. The procedure traditionally employs an invasive approach to assess the nucleic acid content of embryos. All biopsy procedures require high technical skill, and costly equipment, and may impact both the accuracy of genetic testing and embryo viability. To overcome these limitations, many researchers have focused on the analysis of cell-free DNA (cfDNA) at the preimplantation stage, sampled either from the blastocoel or embryo culture media, to determine the genetic status of the embryo non-invasively. Studies have assessed the origin of cfDNA and its application in non-invasive testing for monogenic disease and chromosomal aneuploidies. Herein, we discuss the state-of-the-art for modern non-invasive embryonic genetic material assessment in the context of PGT. The results are difficult to integrate due to numerous methodological differences between the studies, while further work is required to assess the suitability of cfDNA analysis for clinical application.

Keywords: Preimplantation genetic testing, embryo biopsy, cell-free DNA, embryo culture medium, blastocoel fluid, non-invasive PGT, blastocyst.

1. INTRODUCTION

Infertility problems, genetic diseases and chromosomal disorders represent major issues in reproduction, to which Assisted Reproductive Technology (ART) and genetic testing contribute substantial solutions with the aim of achieving a healthy live birth. Currently, the ratio of babies conceived by ART is estimated at 1% of total births in the US and approximately 3% in Europe (possibly higher in some European countries) [1, 2]. Despite significant advances since the first *in vitro* fertilization (IVF) cycle in 1977, ART still has a relatively low success rate with fewer than 30% of initiated cycles leading to live birth [2].

Genetic testing is nowadays used to 1) define the genetic causes of infertility, 2) identify couples at risk of transmitting a genetic disease to their offspring *via* carrier screening, 3) reduce the chance of an affected pregnancy for high-risk couples, *via* ART combined with Preimplantation Genetic Testing (PGT) for monogenic disease (PGT-M) or for structural rearrangements (PGT-SR), 4) reduce the chance of an affected child *via* Prenatal Diagnosis, but also 5) improve ART success (*i.e.* increase implantation and live birth rates and reduce miscarriage rates). The latter is approached through screening embryos created by ART for chromosomal abnormalities (an approach known as PGT for aneuploidies, PGT-A), with the aim to detect and transfer a euploid embryo to the womb.

PGT-A has been applied clinically since 1992 and despite its extensive application worldwide, it is still debated whether it increases implantation and live birth and reduces miscarriage rates [3-5].

Scientific disputes and ethical dilemmas have characterized the PGT field through the years, provoked by the application of controversial indications such as PGT for HLA-typing, non-life-threatening disorders, cancer predisposition, late-onset disorders, or more recently PGT for polygenic disease [6-10].

Along with the varying ethical perspectives, the methodological approaches for genetic analysis in PGT have greatly evolved over time, as have the procedures for sourcing embryonic DNA for testing.

In particular, PGT-M has evolved from using targeted simple or nested polymerase chain reaction (PCR) protocols to multiplex PCR (for amplification of the pathogenic variant region along with informative short tandem repeats - STRs) and Whole Genome Amplification (WGA). WGA

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facilitates numerous subsequent PCR assays, or single nucleotide polymorphism (SNP) arrays, and/or next generation sequencing (NGS) [11]. This advance has enabled a transition from family-specific towards generic protocols, decreasing protocol work-up time and hence time from referral to treatment.

Chromosomal analysis initially involved the use of Fluorescent *In Situ* Hybridization (FISH) for testing a subset of chromosomes but quickly moved to new diagnostic techniques that enable comprehensive chromosomal analysis, such as array comparative genomic hybridization (aCGH), quantitative PCR (which amplifies a limited section of each chromosome), SNP arrays and NGS [12].

The more recent technological advancements (SNP arrays and NGS) enable concurrent monogenic and chromosomal assessment with improved accuracy and cost efficiency. At the same time, however, these technologies have increased the volume of genetic information potentially derived from each sample, often complicating the evaluation and reporting of PGT results. Such information includes higher resolution of mosaicism, structural aberrations, segmental abnormalities, chromosomal deletions, uniparental disomy, polyploidy, as well as analysis of mitochondrial DNA (mtDNA) [13].

Chromosomal mosaicism has been the most challenging to translate into clinical utility. The true incidence of mosaicism in preimplantation embryos is difficult to evaluate due to technical and biological limitations. When mosaicism is detected, clinical management remains unclear, as the result may not truly reflect the developmental potential of the embryo and it is difficult to predict any risk, phenotype and long-term effect on the offspring [14-16].

Good practice recommendation documents and position statements relevant to all PGT practice have been published by major scientific societies to assist in addressing the emerging diagnostic and reporting challenges [17-20].

The scientific and clinical communities have embraced the improved technologies for genetic testing and continue to identify unmet needs and new goals in PGT.

In this manuscript, we aim to compare and contrast the traditional and emerging approaches to PGT, with a focus on the latest strategies for sampling embryonic DNA, to provide an update on data and parameters inspiring further research.

2. TRADITIONAL AND MODERN SOURCES OF EMBRYONIC DNA

2.1. The Biopsy Procedure

Preimplantation genetic testing has traditionally employed an invasive approach (biopsy) to access the nucleic acid content of embryos. The first step in all biopsy procedures involves creating an opening through the zona pellucida (ZP), the thick membrane that surrounds the growing oocyte and the preimplantation embryo until the expanded blastocyst stage, in order to remove material for testing (biopsy). This can be performed mechanically or chemically but the most common approach today involves use of a non-contact laser beam [21].

Biopsy may involve: a) the oocyte polar bodies, which are the byproducts of oocyte meiosis. The first polar body is naturally extruded by the mature oocyte and the second one following fertilization (day 0), b) one (or two) blastomeres at the cleavage stage, on day 3, when the embryo comprises of 6-8 cells and/or c) embryonic cells at the blastocyst stage, usually on day 5. At this stage, the first lineage specification has occurred and the embryo has differentiated to the trophectoderm (TE), an outer layer of epithelial cells that will give rise to the placenta, the compact inner cell mass (ICM) that will give rise to the fetus and yolk sac, and a fluid filled cavity, the blastocoel. High-quality blastocysts comprise 160 to over 200 cells. Researchers have concluded that the most suitable number of TE cells to biopsy is 5-10 [22, 23]. Although some issues relevant to the functions of the trophectoderm and the potential impact of TE biopsy on the inner cell mass and subsequent implantation remain unknown, TE biopsy has currently become the most commonly practiced technique today [10, 24].

Biopsy of day 4 embryos (morula), prior to the blastocyst stage, has also been described and permits the biopsy of more than one cell leaving time for genetic analysis and fresh embryo transfer [25]. Reports on this approach are limited and there remains uncertainty regarding the fate of cells retrieved and how biopsy at this embryonic stage may affect development and implantation [26]. Furthermore, the reproducibility and safety of this technique have not been systematically investigated and the procedure has seen very limited application to date [21].

Overall, the latest follow-up results comparing outcome data (developmental neurological and cognitive assessment, psychomotor and social functioning) from children born following PGT at different biopsy stages have so far been reassuring, but further monitoring of the safety of PGT and the long-term health of children remains necessary [11, 27-30].

Notably, all biopsy procedures have been associated with advantages and limitations relevant to the accuracy of genetic testing but also the impact on embryo viability (Table **1**).

Polar body biopsy has been associated with increased rates of embryo fragmentation and developmental arrest. Day 3 biopsy has been associated with poor subsequent embryo development, with lower implantation potential, when compared to TE biopsy, depending on the number of cells biopsied [31-38]. In animal models, blastomere biopsy has also been associated with epigenetic changes in the resultant offspring [39]. Blastocyst biopsy carries several advantages in comparison to the other procedures, as indicated in Table **1**, and is generally considered less detrimental to the embryo, although several adverse effects have also been noted. A negative impact on implantation is associated with the number of cells removed and the developmental status of the blastocyst at the time of biopsy [40-44]. It has also been speculated that the potential damage caused to the embryo, along with the aforementioned inability of a few cells to predict the ploidy of the whole embryo due to mosaicism, may be responsible for the inability of recent studies to prove a clinical benefit of the latest version of PGT-A (TE biopsy and NGS) [45]. Finally, recent studies have associated TE biopsy with a significant increased risk of preeclampsia and hypertensive disorders of pregnancy [46, 47].

Table 1. Advantages and limitations of current biopsy methods in PGT.

-	-	**Polar Body Biopsy**	**Cleavage Stage Biopsy**	**Blastocyst Biopsy**
General Characteristics	May be considered the least invasive biopsy approach for PGT and may be the only option in cases where embryo genetic testing is not permitted	✓	-	-
	Most labor-intensive and time-consuming approach	✓	-	-
	Indirect approach for obtaining genetic information on the growing embryo	✓	-	-
	Genetic analysis may be provided with a single biopsy sample	-	✓	✓
	Involves testing of fewer embryos	-	-	✓
	Allows less time for genetic analysis prior to fresh embryo transfer	-	-	✓
	May not be compatible with fresh embryo transfer so may require embryo cryopreservation	-	-	✓
Genetic Analysis	Permits evaluation of the maternal genome only (*i.e.*, provides no information on paternal contribution, or meiotic/mitotic errors that may arise post-fertilization)	✓	-	-
	Allows determination of embryo sex	-	✓	✓
	Provides the highest DNA input for genetic analysis, and hence is associated with reduced risk of allele dropout (ADO), amplification failure or inconclusive results and increased diagnostic accuracy	-	-	✓
	Associated with biological and technical limitations due to chromosomal mosaicism (which may impact the reliability of genetic analysis)	-	✓	✓
	Enables detection of chromosomal mosaicism	-	-	✓
Procedure-Related Risks	May impact embryo development, implantation potential or live births	✓ [34]	✓ [31-33, 35-38]	✓ [40-43]
	Carries risks associated with prolonged culture	-	-	✓ [48]
	Carries risks associated with cryopreservation	-	-	✓ [49]
	Has been associated with pregnancy complications, maternal disease or adverse perinatal outcomes (birth weight, gestational age, morbidity)	-	✓ [30]	✓ [46, 47]
	Has been associated with reassuring neonatal outcomes	-	✓ [11, 29]	✓ [27, 28]

Biopsy procedures also require high technical skills as well as costly equipment and increase considerably the workload in the embryology laboratory. The biopsy step may present a "bottle-neck" as demand for PGT rises, for example, with the identification of more at-risk couples due to the expanding application of carrier screening [50].

To overcome the limitations associated with the invasive biopsy procedure, many researchers have focused on the collection and analysis of cell-free DNA (cf-DNA) at the preimplantation stage, in an attempt to determine the genetic status of the embryo non-invasively. This approach is referred to as non-invasive PGT (niPGT).

2.2. Access to Embryonic Cell-free DNA At the Preimplantation Stage

Cell-free DNA fragments are present in body fluids associated with vesicles, such as extracellular vesicles or apoptotic bodies, or in a free circulating form so analysis can be performed by minimally invasive or completely non-invasive methods.

In the fields of reproductive medicine and ART, cfDNA profiling has been studied in the male and female reproductive system (seminal plasma, follicular fluid (FF) or maternal serum) and the preimplantation embryo. There are indications that parental cfDNA can be used as a biomarker of semen and oocyte quality, male and female infertility, response to stimu-

lation and pregnancy outcome, while embryonic cfDNA has been assessed within the context of PGT for the detection of embryo genetic disorders and assessment of chromosomal status [51]. Embryonic cfDNA is released and transferred through the zona pellucida, along with other substances (carbohydrates, amino acids, autocrine and paracrine growth factors, microRNAs and proteins), and serves in communication between the embryo and its environment (*e.g*. the oviduct or the endometrium) throughout embryo development or the initiation of implantation. Research has shown that extracellular vesicles secreted by blastocysts may be taken up by endometrial epithelial cells [52, 53].

Aspiration of embryonic cfDNA from blastocoel fluid with an ICSI pipette piercing through the trophectoderm layer, termed blastocentesis, was first reported by Palini *et al.* in 2013. Embryonic cfDNA also collects in the culture media in which the embryo grows during IVF; Spent Culture Media (SCM) cfDNA sampling was first demonstrated by Shamonki *et al.* in 2016 [54, 55].

Blastocentesis may be easily implemented in a clinical setting as collapse of the blastocoel fluid cavity is often performed prior to embryo vitrification (a cryopreservation method) in order to prevent crystal formation. It requires, however, special embryological skills, exposes the embryo to suboptimal environmental conditions and remains a semi invasive procedure as the embryo is manipulated even though embryonic cells are not removed [56].

In contrast, sampling of SCM is completely non-invasive. Embryonic cfDNA has been detected in culture media as early as days 2 and 3 and its amount increases over subsequent days. In the first study to attempt this approach, culture media was collected from embryos grown from day 3 to 5/6 (blastocyst stage). Only 2/55 adequately amplified samples yielded a reliable niPGT-A result (corresponding to TE biopsy), but technique performance has significantly improved since that first application [55].

3. CELL-FREE DNA OF THE PREIMPLANTATION EMBRYO: FACTS AND DATA

Research on niPGT has provided some insights into the origin and role of embryonic cfDNA but has also given rise to contradicting data as well as concerns relating to the potential clinical utility of this approach.

cfDNA is released in the process of embryo-environment communication, but also as a consequence of apoptosis which serves to regulate cell numbers during development or eliminates abnormally developing cells (self-correction) [57-59].

Table **2** summarizes the main findings in the literature relevant to the origin of cfDNA.

Embryonic self-correction was first demonstrated in mouse embryos with mosaicism induced using a spindle checkpoint inhibitor during division [71]. The authors observed proliferation of euploid cells in the TE and active

Table 2. Findings relevant to cfDNA release and its correlation with embryo quality and viability.

Research Findings	Evidence	References
Embryonic cfDNA is released as a consequence of apoptosis	A positive correlation has been observed between caspase protease activity and cfDNA levels.	[57]
	BF contains DNA fragments of 160-220bp and 300-400bp size, consistent with apoptosis (revealed by NGS analysis).	[58]
Human embryos have the ability to self-correct by eliminating aneuploid cells, cell debris and fragments	Embryos with mosaicism are able to implant and lead to live birth.	[16]
	Human blastocysts eliminate cell debris with abnormal chromosomal rearrangements.	[26]
	Mosaic embryos form partially compacted morulas and exclude aneuploid cells.	[59]
	BF of euploid blastocysts indicates higher amplification failure than BF of aneuploid blastocysts (ploidy determined by TE biopsy).	[60]
	The amount of cfDNA in BF and culture media is related to embryo quality and ploidy.	[57, 61, 62]
Additional mechanisms (besides apoptosis) may drive cfDNA release	More advanced blastocysts (with higher number of cells, fully expanded), are recognized to have increased rates of cfDNA in the blastocoel cavity, when compared to those with delayed development, and successful amplification of cfDNA is also more likely.	[61, 62]
	SCM cfDNA results may be highly concordant (>90%) to TE, ICM and whole embryo, regardless of embryo quality or chromosomal status. The SCM-ICM concordance has been found to be similar to TE-ICM from the same blastocysts.	[60, 63-65]
	Similar quantities of BF and SCM cfDNA and sizes of amplified fragments were obtained among embryos of different quality and ploidy.	[58, 66-69]
	Higher amplification failure has been observed on BF cfDNA analysis from embryos leading to successful pregnancy. Low embryonic cfDNA in SCM has been associated with healthy pregnancies and live births (higher copy number associated with impaired intrauterine development and miscarriage).	[60, 70]

Note: BF: blastocoel fluid, SCM: spent culture media, ICM: inner cell mass.

elimination of aneuploid cells by apoptosis in the ICM. In addition, aneuploid cells in post-implantation mouse embryos have been found to be effectively eliminated in the developing epiblast compartment by autophagy and apoptosis, while chromosomally normal cells compensate for this loss by increased proliferation [72]. Depletion of aneuploid cells from the extraembryonic germ layer and proliferation of euploid cells was also recently demonstrated in human embryos and gastruloids [73]. Furthermore, studies on human embryos have indicated that the proportion of aneuploid-to-euploid cells within mosaic embryos (*i.e*. the "level of mosaicism") decreases throughout development such that a high proportion of embryos that were mosaic in their early stages remain viable and present with euploid profiles after extended culture and through the peri-implantation stages (up to 12 days post-fertilization) [14, 74]. A recent single-cell analysis at the blastocyst stage (using single cell RNA-seq data) did not detect an increased number of aneuploid cells in the TE in comparison to the ICM, but after *in vitro* culture to the post-implantation stage, aneuploidy was more frequently detected in the extraembryonic trophoblast compartment [75]. It seems that placental tissues have a higher tolerance for cells with aneuploidy. Persistence of aneuploid cells in the extraembryonic tissues gives rise to confined placental mosaicism, which is observed in about 2% of prenatal trophoblast samples undergoing karyotype testing [76].

With regards to the accuracy and clinical value of employing cfDNA analysis for the assessment of embryonic status during niPGT, several concerns have been raised:

1) Embryos with high implantation/pregnancy potential may be disposed. Preferential elimination of aneuploid cells, in the process of self-correction, may lead to false positive results from the analysis of cfDNA. Studies are yet to prove how well the cfDNA analysis reflects the genetic status of the whole embryo. Recently, Chen *et al.* provided insights into the characterization of cfDNA in culture media by employing single cell whole genome DNA methylation sequencing. From their assessment of day 6 spent culture media samples with no cumulus cell or polar body contamination (as established by single cell methylation profiling) the authors concluded that approximately two-thirds of the samples were positioned with the epiblast DNA methylation profile and one-third of samples were positioned with the TE profile, suggesting that cfDNA in culture media can be derived from both the TE and ICM [77].

2) Results may not indicate purely embryonic DNA due to the high risk of contamination associated with these analyses. The percentage of embryonic cell free DNA identified in culture media, as determined in one of the earlier studies, ranges significantly (0-100%), with a median fraction estimated at 8% due to the high percentage of maternal DNA also detected [67]. Most studies have failed to confirm that their results are based purely on embryonic DNA. Contamination may originate from the IVF culture media, exogenous sources (lab personnel), sperm cells, or most commonly due to the breakdown of maternal cumulus cells and even polar bodies that may remain attached to the ZP and may even persist to the blastocyst stage [77, 78]. Several measures are recommended to minimize the risk of contamination including careful handling, testing media for contamination prior to use and change during culturing, oocyte denudation and use of Intracytoplasmic Sperm Injection (ICSI), to minimize the risk of cumulus cell and sperm contamination, and handling of embryos individually with dedicated single-use capillaries.

3) Current non-invasive sampling procedures may be damaging to the embryo. Studies on non-invasive PGT have, thus far, involved some deviation from routine IVF and embryo handling procedures (penetration of the TE layer during blastocentesis, vitrified-warmed embryo culture, assisted hatching, washing in several media drops or embryo biopsy) or from currently validated protocols (employing culture drops of reduced volume or culturing for an extended length of time), in an attempt to improve the yield of cfDNA sampling. The above may have an impact on DNA quality but also compromise embryo viability. Some reassuring data for the above has become available, for example, in finding that different culture systems, equipment, and materials (incubators and brands of culture media) do not interfere with the results and that small culture volumes do not affect embryo development [63, 79, 80]. In addition, although extended culture and vitrified-thawed embryo transfers have been associated with improved clinical outcomes, extended cryopreservation time has also been reported to have an adverse impact on live births [49].

4. A LOOK INTO THE STRATEGIES EMPLOYED FOR NIPGT

niPGT has been attempted for PGT-A and, to a lesser degree, PGT-M using BF-sampling, SCM sampling or a combination of these approaches.

The results of the available niPGT studies are very difficult to integrate due to methodological differences, such as the type of embryo culture (single-step, continuous, or sequential), drop culture volumes, measures taken to reduce contamination, storage conditions, timing and length of media exposure to the embryo, volume of BF/SCM sample tested as well as technicalities and different analytical approaches (inclusion of a cell lysis/DNA extraction step, selected whole genome amplification method, downstream testing and diagnostic algorithms for interpretation of results). Recent reviews have attempted to provide an in-depth discussion of these different approaches and provide recommendations for what seems to be the most valid methodology [65, 81].

A brief summary of non-invasive PGT-M and PGT-A studies follows below.

4.1. Non-invasive PGT-M

4.1.1. Blastocoel Fluid (BF) Sampling for PGT-M

Limited data is available with regards to BF-sampling as a basis for genotype assessment of the preimplantation embryo. The few available studies have assessed cfDNA on a very limited number of samples, either directly with the use of quantitative PCR (custom or validated TaqMan genotype assays) for the amplification of multicopy genes on chromo-

somes 17 and Y or the combined detection of specific gene variants and multiple SNPs surrounding them [61, 82]. In other studies, whole genome amplification (PicoPlex or RepliG multiple displacement amplification, MDA) was followed by PCR for the amplification of specific regions of selected genes (*TCIRG1, SCN5A, RHO, EXTL1, SLC4A1, VWF, HSF4, NPC1, PTCH1, EPS8L3, SMN1, SRY, ACTB, PAH*) and validation of a couple of these by sequencing [58, 82, 83]. These studies, reviewed and summarized in a table in Leaver and Wells 2020, have demonstrated the possibility of cfDNA detection in BF but also underlined the limitations associated with variable and low detection rate, even after WGA, ranging from 42.9-84% (much lower in comparison to the ~95% efficiency WGA efficiency achieved from biopsied cells), increased allele dropout, risk of maternal contamination, lack of consistency and reliability [84].

4.1.2. SCM Sampling for PGT-M

Direct assessment of SCM samples, without prior WGA, has employed qPCR mainly for sexing, which involved amplification of the *SRY* or *TSPY1* genes along with controls such as Alu repeat sequence or a multicopy gene, *TBC1D3* [82, 85, 86]. Amplification of 378 loci (mutation sites and SNPs) has also been reported, with the use of TaqMan genotype assays, in a study mentioned above that also performed BF cfDNA testing [61]. Nested qPCR has also been performed for the detection of a specific alpha-thalassaemia deletion common in Southeast Asia [87].

Assessment of SCM samples has also involved WGA methods (PicoPlex and MALBAC) followed by PCR and sequencing for genotyping of the *MTHFR* rs1801133 polymorphisms, *HBB* pathogenic variants (beta-thalassaemia) (Sanger sequencing) and linked SNPs (NGS) [82, 88, 89].

The above studies all highlight the need to prevent maternal contamination, which interferes with SCM analysis for PGT, and agree that diagnostic efficiency is improved when sampling is performed at a later culture day, with more robust results confirmed on day 5 of culture. Maternal contamination and low concordance with biopsied cells remain unresolved issues. Finally, a more recent study by Esmaeili *et al*. 2022, reported that issues with contamination in PGT-M may be minimized by assessment of RNA rather than DNA. In their study, they investigated an approach for non-invasive sexing by use of PCR and reverse transcriptase-PCR (RT-PCR) based on the presence of SRY DNA and RNA in SCM (with *GAPDH* as positive control) and determined that RNA amplification methods may be more reliable in comparison to DNA testing, the latter leading to more misdiagnoses due to contamination [90].

Details of the above studies reporting SCM sampling for PGT-M are included in Table **3**.

In conclusion, current data on non-invasive PGT-M is scarce and further work is required to assess the feasibility of clinical application.

4.2. Non-invasive PGT-A

Overall, niPGT-A has been more extensively investigated in comparison to niPGT-M. The numerous studies reported have multiple differences between them, with regards to type and quality of samples, day of sampling, sampling volume, embryological handling procedures, PCR amplification techniques, WGA methods and analysis platforms. Extreme inconsistencies have been reported even across studies that employ the same methodology. These may be related to inter-laboratory differences, for example relevant to experience in performing the required procedures or the detection and reporting of mosaicism. Notably, such variability has also been observed for invasive PGT-A. A brief up-to-date summary of niPGT-A studies is provided below.

4.2.1. BF Sampling for PGT-A

Following the first demonstration, in 2013, of the possibility to determine gender and detect aneuploidy in only two BF samples with the use of Repli-G WGA and aCGH, four subsequent studies from 2014-2018 reported BF niPGT-A results with Sureplex WGA and aCGH analysis and estimated whether the chromosomal status corresponded with ploidy in TE, whole embryo, polar body and blastocyst [54, 56, 60, 62, 91]. Results varied amongst these studies, with successful WGA confirmed for 63 to 87% of samples. Concordance with the trophectoderm biopsy was deemed high, but also ranged from 37.5% to 97.4%. Two subsequent studies compared BF cfDNA results with TE biopsy by Sureplex or Picoplex followed by NGS analysis, though in one of them, the methodology for TE analysis differed from the one employed for niPGT [12, 61]. In these, successful amplification was detected in 34.8% and 87.4% of samples, respectively, while concordance with TE results was 37.5% and 40% respectively for each study. The above studies are reviewed and summarized in a table in Leaver and Wells 2020 [84]. A common theme amongst some of these studies is that mosaicism was more apparent in BF cfDNA compared to results from embryonic cells. This may indicate that aneuploid cells are marginalized into the blastocoel cavity to support the formation of a resulting euploid blastocyst.

4.2.2. Spent Culture Media (SCM) for niPGT-A

Numerous studies have involved SCM sampling followed by WGA and chromosomal investigations. A variety of WGA techniques have been tested, such as MALBAC, Sureplex, Picoplex or modified approaches (double WGA, modified MALBAC), followed by aCGH or NGS analysis and comparison of results with TE biopsy, whole embryo or PB biopsy (the latter in one study). The most commonly reported approach involves MALBAC followed by NGS [63, 68, 69, 88, 92-104]. In all these studies, which are summarized in Navarro-Sanchez *et al.*, 2022, amplification success ranges from 62.7% to 100%, but concordance with TE biopsies varies and depends on whether ploidy concordance or full chromosome concordance is reported [65]. The majority of these studies have involved embryological manipulations, such as vitrification or assisted hatching, media change and embryo biopsy. The largest study to date is the one reported by Rubio *et al*. in 2020, which involved 1301 samples, with no manipulation to the embryos but extended culture to culture day 6/7 and culturing in a small media volume [63]. In this study, successful amplification was reported to be 85.2%, general euploid/aneuploid concordance 78.2%, and full concordance 67.7%.

Table 3. Use of spent culture media as a source for niPGT-M.

Reference	Samples	Embryo Manipulation	Culture Volume (µl)	Day of Sampling (Number of Samples)	Volume of Media Analyzed (µl)	Methodological Approach and Target	Target	Observations-Comments
Direct Assessment of SCM (without WGA)								
[82]	86 SCM from embryos of IVF couples	None	10	D3 (32) D5/6 (54)	2	qPCR for multi-copy genes	Multi-copy genes *TBC1D3*, *TSPY1* (Chr 17 and Y)	Higher DNA content on day 5 *vs* day 3, poor amplification of *TSPY1* before day 5, confirmation of accurate sex determination for seven SCM samples (comparison with 2 TE biopsies and 5 pregnancies)
[87]	202 SCM from 38 couples undergoing a-thalassemia PGT and 6 IVF couples requiring ICSI	ICSI Day 3 biopsy	50	D5/6 (413) D1 to D6 sampling (148)	5	Nested qPCR	a-thalassemia, South-East Asian deletion genotype	Better diagnostic efficiency from SCM compared to biopsy-based fluorescent gap PCR analysis (88.6% *vs* 82.1%) Diagnostic efficiency from SCM increases with day of culture, optimum being on day 5
[86]	26 SCM of IVF couples Paired embryo-medium samples tested for different days of culture media sampling	ICSI Assisted Hatching (AH)	12.5	D1 (5), D2 (4), D3 (9), D4 (4), D5 (5), D6 (4), D6 with AH (3)	10	PCR and electrophoresis on 2% agarose gels	*SRY*	Successful *SRY* amplification only from D3 to D6 samples (not from earlier sampling) Accurate sex detection in D3 to D6 discarded embryo and corresponding medium Stronger positive signal for D6 blastocysts following AH and corresponding medium
[61]	72 SCM from 14 PGT couples and corresponding TE biopsies - comparison with 69 Blastocoel fluid and corresponding TE samples	None. SCM collected before biopsy	25	D5 (72)	25	qPCR (TaqMan genotyping assays)	Pathogenic variant and linked SNPs (378 loci)	2.9% (2/69) of BF samples and 20.8% (15/72) of SCM samples concordant with the corresponding TE High risk of maternal contamination in SCM
Assessment Following WGA								
[82]	8 SCM from blastocysts of IVF patients known to carry the target polymorphism	None.	10	D5/6 (8)	2.5	PicoPLEX (Rubicon Genomics) and PCR/direct sequencing	*MTHFR* rs1801133	*MTHFR* amplification not successful without WGA. Genotyping success: 63% (5/8), concordance confirmed for one of two available TE biopsies (possible allele drop-out in the second SCM sample)

(Table 3) contd….

Reference	Samples	Embryo Manipulation	Culture Volume (µl)	Day of Sampling (Number of Samples)	Volume of Media Analyzed (µl)	Methodological Approach and Target	Target	Observations-Comments
Assessment Following WGA								
[88]	88 SCM from 7 PGT couples, including 10 embryos - from 1 couple undergoing PGT-M for beta thalassemia	ICSI, embryo biopsy	30	D5 (10 for PGT-M)	30	MALBAC (Yikon Genomics)-PCR and NGS	HBB pathogenic variant and 60 linked SNPs	Overall 92.8% successful WGA from blastocyst SCM, lower amplification efficiency from SCM of poor quality samples, 100% successful detection of the pathogenic variant (in 8/8 amplified SCM), SNP data validated concordance between medium and embryo results in 8/8 samples
[90]	71 embryos from PGT couples	ICSI, Group 1: SCM from 40 embryos after biopsy, Group 2: 31 SCM from non-biopsied embryos	10	D5 (71)	10	RNA extraction (ExoRNeasy Serum/plasma kit (Qiagen, USA)), cDNA synthesis miscriptII RT kit(Qiagen, USA), PCR/RT-PCR	*SRY* DNA/RNA (*GAPDH* as control)	100% diagnostic accuracy in group 1 (comparison with corresponding FISH data) *vs* 83.8% in group 2. RNA amplification methods appear more reliable than nucleic acid (due to contamination issues), with high sensitivity (100%), specificity (94.44%) and diagnostic accuracy (96.88%)
[89]	59 SCM samples from 10 couples undergoing PGT-M for beta-thalassemia	ICSI, Group A: 26 samples containing BF (following embryo biopsy) Group B: 33 samples without BF	25	D5 (59)	10 (of stored mixture of 20µl culture media and 5µl lysis buffer)	MALBAC, Sanger sequencing and NGS haplotyping	*HBB* pathogenic variant and 90 linked SNPs	Overall higher diagnosis rate in group A (80.8%) *vs* Group B (51.5%), 100% concordance with biopsy results in group A

Note: SCM: spent culture media, RT-PCR reverse transcriptase-PCR, AH: assisted hatching, SNP: single nucleotide polymorphism, FISH: fluorescence *in situ* hybridization.

Even though no approach has been standardized yet, clinical application has been reported in a few studies/case reports either in combination with TE biopsy or even as a sole genetic test, while some IVF centers are already offering niPGT-A as an add-on to IVF cycles. Xu *et al.* (2016) employed their niPGT-A approach (MALBAC, NGS) for screening embryos from 7 couples, of which 6 achieved clinical pregnancies leading to five live births at the time of publication [68]. Fang *et al.* (2019) employed MALBAC with NGS for testing of 170 blastocysts, detecting 79 euploid embryos, leading to transfer of 52 embryos, a clinical pregnancy rate of 58% and 27 babies delivered [93]. Franco *et al.* (2020) reported the first occurrence of child-birth following niPGT-A in Brazil [95]. Details of selected SCM niPGT-A studies with available information on reproductive outcome are provided in Table **4**.

A few studies have combined BF and SCM sampling for PGT-A and recently for PGT-M, which represents an interesting option and may provide improved amplification and concordance results, but still requires micromanipulation for BF sampling [66, 89, 105-108].

Finally, chromosomal mosaicism has been reported in SCM during niPGT-A, with an incidence corresponding to that detected during invasive PGT-A. The majority of "mosaic" results indicated high-level mosaicism, which may relate to the process and purpose of embryonic self-correction mechanisms. Similar to invasive PGT-A, mosaicism did not vary depending on maternal age, while aneuploidy levels had a positive correlation with maternal age

Table 4. A summary of selected studies on SCM niPGT-A in ICSI cycles with known reproductive outcome.

Reference	Subjects (Maternal Age)	Indication	Manipulation	Culture Volume (µl)	Day of Media Sampling	Volume of Media Analyzed (µl)	Methods	Case Results	Clinical Pregnancy Rate per Transfer Cycle	Miscarriage Rate per Pregnancy Achieved	Live Birth per Transfer Cycle
[68]	Seven IVF couples, 26-34 yrs (average 30 years)	Azoospermia, RPL, RIF, abnormal karyotype (inversion, reciprocal translocation, 47 XXY)	ICSI (additional manipulations, such as day 3 freezing of donated embryos, thawing and washing, performed during validation work)	30	D5	5-20	MALBAC WGA (Yikon Genomics) and NGS (Illumina HiSeq 2500 platform) (validation data from 42 SCM samples and corresponding donated whole embryos included in the study)	Pregnancy achieved for six out of seven couples	66.7% (6/9 single embryo transfers)	-	55.55% (5/9) with one pregnancy on-going
[93]	45 IVF couples (23-42 years, average 30)	Group A: 23 couples with chromosomal rearrangements in one partner Group B: 22 couples with normal karyotype	ICSI	30	D5	20-25	MALBAC NGS Illumina HiSeq 2500 platform (validation data as in Xu *et al*. 2016)	413 embryos, 170 blastocysts, 46.5% euploid, 30.6% aneuploid, 19.4% mosaic, 3.5% no result, 52 euploid embryos transferred in 43 women. The two groups did not differ in the rate of ploidy abnormalities	58% (29/50) [52% in Group A, 64% in Group B]	10.3% (3/29) (all three miscarried fetuses were euploid)	54% (27/50), 26 deliveries, 27 healthy babies born. Sex ratio consistent with ni-PGT results
[95]	Case report: 37yr old mother	8-year history of secondary infertility	ICSI	20	D5	20	MALBAC NGS, Illumina MiSeq® platform.	8 embryos, two cryopreserved following SCM collection, one reported euploid and one mosaic, both transferred and implanted, the mosaic arrested at 7 weeks. Birth of a baby girl.			
[70]	40 SCM, average maternal age 35yrs.	Routine IVF cycles	ICSI Assisted hatching on day 3	40ul (20µl spent media stored on day 3)	D3	5	MALBAC, NGS Illumina HiSeq 4000 utilising	SCM collected from 542 morphologically good quality embryos transferred with follow-up. Comparison of 20 SCM from embryos that miscarried to 20 SCM from embryos leading to healthy neonates. Low SCM gDNA was found to be characteristic of live birth. Clinically significant chromosomal ploidy alterations were found only among SCM corresponding to aborted embryos.			

Note: RPL: recurrent pregnancy loss, RIF: recurrent implantation failure, ICSI: Intracytoplasmic Sperm Injection. MALBAC: multiple annealing and looping-based amplification. WGA: whole genome amplification.

[109]. In another recent study, SCM niPGT-A results were also investigated relevant to pregnancy outcome, and live births were associated with low embryonic cfDNA in SCM [70]. This last study also incorporated the analysis of the chromosomal status of embryos that miscarried, where multiple chromosomal ploidy alterations, incompatible with embryo viability, were detected by NGS and Copy Number Variation (CNV) evaluation.

Use of cfDNA for PGT has distinct benefits and drawbacks. The diagnostic accuracy of niPGT has not outperformed the results obtained following conventional trophectoderm biopsy, protocol validation remains in progress, and safety issues are under debate.

A double-blinded multicentre randomised controlled trial is currently ongoing to compare the ongoing pregnancy rate after embryo transfer selected by non-invasive preimplantation genetic testing niPGT *versus* conventional morphological evaluation [110].

Overall, niPGT is not widely deemed to have reached a standard required for primary clinical application. A recent study suggested its use as a screening method complementary to TE biopsy, based on results indicating that transfer of embryos with both TE and SCM results indicating euploidy were associated with higher implantation rates and no miscarriage [101]. This, however, defeats the original purpose of niPGT, which was to provide non-invasive genetic testing as a sole approach, without manipulating the embryo.

5. FURTHER POTENTIAL FOR NON-INVASIVE ASSESSMENT OF EMBRYONIC GENETIC MATERIAL (DNA/RNA)

A few studies to date have investigated the secretion of microRNAs from embryos to their external environment and demonstrated their presence in both BF and SCM. MicroRNAs are single-stranded non-coding RNA molecules of 22 nucleotides in length that function as regulators of gene expression. Embryonic microRNAs can be free or enclosed in extracellular vesicles, which are secreted by human embryos at different developmental stages and can enter endometrial cells *in vitro*, coordinating in this way communication between the embryo and the endometrium and the process of implantation [53, 111]. A few studies to date have drawn attention to the potential use of microRNAs as biomarkers of implantation, chromosomal status and pregnancy outcome [112-117]. However, there is a great discrepancy between results and the relevance of released microRNAs as biomarkers has been questioned. The main limitations involve the small amount of starting material available, as well as the detection of nonembryonic microRNAs in commercial culture media. For further investigations, researchers have highlighted the importance of a later day of media sampling, employing more sensitive techniques (*e.g*., NGS, digital PCR), and, most importantly, adequate methodological strategies for data analysis [116, 118].

Fragmented tRNAs, 35nt molecules produced by cleavage of the full length tRNAs, and long non-coding RNAs, RNAs longer than 200 nucleotides, have also been proposed as potential tools for non-invasive assessment and their role in early human development merits further investigation [118-120].

Finally, some studies have focused on mitochondrial DNA (mtDNA) which has been detected in SCM from day 3 embryos [121]. Levels of cell-free mtDNA have been correlated with embryo fragmentation rate, developmental competence, TE quality, implantation rate, morphokinetics [122-124].

CONCLUSION

The future holds great promise for the ART/PGT field. The latest methodological advances and improved bioinformatic approaches expand the application of preimplantation testing, further improve accuracy and reliability of both monogenic and chromosomal analysis and may also provide additional information (*e.g*. combined low-pass whole genome sequencing with transcriptomic profiling) to improve not only embryo selection but also our understanding of critical biological processes at this stage of development [125-128].

Further work is required to support the clinical applicability of niPGT and achieve the benefits of non-invasive testing. Perhaps a focus on extracellular vesicles will be of use, as work in other fields (oncology) has also demonstrated that these may have a higher cfDNA level than the free circulating form and have been associated with improved sensitivity and specificity at genotyping [51, 129-131].

Finally, reference must be made to the numerous additional methodologies that involve the profiling (genomic, transcriptomic, proteomic or metabolomic) of follicular fluid, oocytes, cumulus cells, embryos or culture media, mitochondrial DNA analysis (embryos or cumulus cells) and more recently genomic profiling of prospective parents [132-141]. Although these approaches have provided some data relevant to oocyte fertilization potential and embryo developmental competence, no single approach has been as yet widely validated and applied to identify embryo(s) with the highest viability towards improving the success of IVF.

Therefore, the search continues for approaches to identify the most viable embryos. Along with the advances in genetic testing, the constant improvements in the medical and embryological fields relevant to both reproduction and assisted reproduction (*e.g*., improved embryo assessment by use of artificial intelligence, improved culture media, progress in understanding of endometrial receptivity and determining optimal timing for embryo transfer), will eventually indicate the factors that must be combined to increase the chance of a healthy pregnancy and live birth.

LIST OF ABBREVIATIONS

aCGH	=	array Comparative Genomic Hybridization
ART	=	Assisted Reproductive Technology
BF	=	Blastocoel Fluid
cf-DNA	=	cell-free DNA
CNV	=	Copy Number Variation
FF	=	Follicular Fluid
FISH	=	Fluorescent *In Situ* Hybridization
ICM	=	Inner Cell Mass
ICSI	=	Intracytoplasmic Sperm Injection

IVF	=	*In Vitro* Fertilization
MDA	=	Multiple Displacement Amplification
mtDNA	=	mitochondrial DNA
NGS	=	Next Generation Sequencing
niPGT	=	non-invasive PGT
PCR	=	Polymerase Chain Reaction
PGT	=	Preimplantation Genetic Testing
PGT-A	=	Preimplantation Genetic Testing for Aneuploidies
PGT-M	=	Preimplantation Genetic Testing for Monogenic disease
PGT-SR	=	Preimplantation Genetic Testing for Structural Rearrangements
qPCR	=	quantitative PCR
RT-PCR	=	Reverse Transcriptase PCR
SCM	=	Spent Culture Media
SNP	=	Single Nucleotide Polymorphism
STR	=	Short Tandem Repeats
TE	=	Trophectoderm
WGA	=	Whole Genome Amplification
ZP	=	Zona Pellucida

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