



Review

# Evolution of Minimally Invasive and Non-Invasive Preimplantation Genetic Testing: An Overview

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**Abstract:** Preimplantation genetic testing (PGT) has become a common supplementary diagnostic/testing tool for in vitro fertilization (IVF) cycles due to a significant increase in cases of PGT for monogenic conditions (PGT-M) and de novo aneuploidies (PGT-A) over the last ten years. This tendency is mostly attributable to the advancement and application of novel cytogenetic and molecular techniques in clinical practice that are capable of providing an efficient evaluation of the embryonic chromosomal complement and leading to better IVF/ICSI results. Although PGT is widely used, it requires invasive biopsy of the blastocyst, which may harm the embryo. Non-invasive approaches, like cell-free DNA (cfDNA) testing, have lower risks but have drawbacks in consistency and sensitivity. This review discusses new developments and opportunities in the field of preimplantation genetic testing, enhancing the overall effectiveness and accessibility of preimplantation testing in the framework of developments in genomic sequencing, bioinformatics, and the integration of artificial intelligence in the interpretation of genetic data.

**Keywords:** blastocoel fluid; blastocentesis; spent culture media; embryo biopsy; mosaicism; next-generation sequencing



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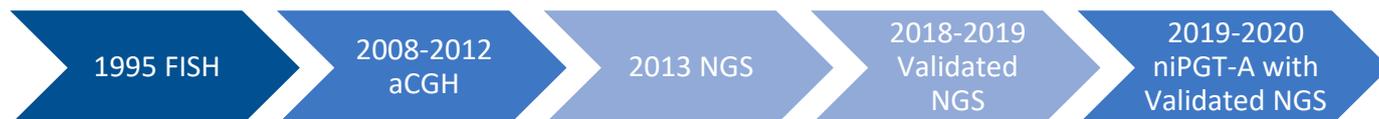
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## 1. Introduction

Preimplantation testing of embryos is an idea that was historically put forward and experimentally tested a few decades before the clinical application of preimplantation genetic testing (PGT) [1]. Although medically assisted reproduction and molecular methods had not evolved up to the standards needed for their clinical application at the time, biopsies were feasible in embryos from experimental animals [2]. The floor was set by the emergence of leading technologies such as FISH and PCR at almost the same time as IVF and, later, intracytoplasmic sperm injection (ICSI) [3]. The ability of both fluorescent in situ hybridization (FISH) and polymerase chain reaction (PCR) to highlight the genetic contents of single or several cells by hybridization and amplification, respectively, met the concept and the criteria for the revolutionary application of PGT.

Later technological advancements in chromosome arrays and deep sequencing shifted the appropriate timeline for embryo biopsy from polar bodies and day-3 blastomeres to the blastocyst stage [4]. Blastocysts became popular at the beginning of the millennium, and it was only a matter of time until blastocyst biopsy took the upper hand in biopsies, shortly after the year 2010 (according to ESHRE data). Several reasons supported the shift towards blastocyst biopsy [5]. The first was the emergence of sequential media, supporting efficient

blastocyst culture with no need to replenish or recondition the medium throughout the culture period [6]. The second was the better selection of compatible embryos for biopsy and the number of trophectoderm cells excised for better and deeper analysis [7]. The third was the advent of multiple parallel sequencing and its potential to extend future applications in chromosome sequences, aneuploidies, and structural variations using the same robust technology for all genetic abnormalities [8]. Consequently, in theory, irrespective of the initial indication for PGT—either monogenic disorders (PGT-M) or structural rearrangements (PGT-SR)—next-generation sequencing (NGS) would result in a diagnostic yield that also covered partial- or whole-chromosome aneuploidies (PGT-A), and vice versa (Figure 1) [9].



**Figure 1.** The schematic progression of PGT-A toward non-invasive and minimally invasive methods is depicted in the diagram below.

**2. Schematic Evolution of PGT-A towards the Minimally Invasive and Non-Invasive Approaches**

The ultimate goal of PGT-A, in particular, was to increase the implantation rate and the live birth rate, reduce the time of pregnancy, decrease pregnancy losses, and precisely diagnose harmful mosaicisms [10]. Although randomized controlled trials (RCTs) and other studies have been conducted to validate the high expectations related to PGT-A, the results were either inconclusive, weak, or negative (Table 1) [11].

**Table 1.** The invasive and non-invasive methods that have been and/or are currently being utilized to assess PGT.

Invasive PGT		
FISH	aCGH	NGS
<ul style="list-style-type: none"> <li>By measuring the copy number of the particular loci, target-specific DNA probes tagged with various fluorochromes or haptens can be used to identify chromosome imbalances linked to meiotic segregation of chromosome rearrangements.</li> <li>Sporadic chromosomal aneuploidy screening in an effort to increase assisted reproduction’s effectiveness.</li> <li>In most cases, predictive value remains low, and routine clinical use is not advised.</li> </ul>	<ul style="list-style-type: none"> <li>Comprehensive examination of an individual’s genome to detect any genetic material gains or losses.</li> <li>Determine whether certain tiny chromosomal regions are duplicated or absent.</li> <li>Green: DNA problem Red: DNA control</li> <li>A DNA “chip” that has a collection of DNA molecules covering the entire human genome is hybridized with a mixture of two fluorescent markers.</li> <li>A scanner examines the hybridization result. Normal DNA content → yellow Green: chromosomal region in excess Red: chromosomal region in defect</li> </ul>	<ul style="list-style-type: none"> <li>Parallel genome sequencing.</li> <li>Facilitates the detection and screening of embryos with chromosomal mosaicism, euploidy, and aneuploidy.</li> <li>NGS-based PGT enhances the success of IVF pregnancy outcomes.</li> <li>Involvement of cleavage-stage biopsies, the transfer of new embryos, and the rate of implantation for recurrent implantation failure (RIF).</li> <li>NGS provide a useful addition to the existing methods for screening for aneuploidy.</li> </ul>

Table 1. Cont.

Minimally invasive PGT	Non-invasive PGT
Blastocoel fluid (BF)	Spent Culture Media (SCM)
<ul style="list-style-type: none"> <li>• Blastocentesis → a minimally invasive procedure.</li> <li>• A puncture of the blastocoelic cavity with an ICSI needle is required to aspirate the fluid during this technique.</li> <li>• The fluid includes embryonic cells and cfDNA. NGS or PCR genetic study of fluid to detect any chromosomal abnormalities.</li> <li>• Compared to embryos with chromosomal defects, euploid embryos have a higher chance of implantation and a lower risk of miscarriage, which is why genetic analysis indicates that euploid embryos are chosen for embryo transfer.</li> </ul>	<ul style="list-style-type: none"> <li>• Embryos cultivated in a particular medium that offers the environment and nutrients required for development. Molecules such as cfDNA, RNA, and metabolites are released into the culture media as the cells grow.</li> <li>• Following around five to six days of embryo cultivation, a small sample is taken from every embryo.</li> <li>• The cfDNA and RNA contained in the medium can be examined by NGS, PCR, or microarray analysis.</li> <li>• During IVF cycles, embryos that are euploid and have appropriate metabolic profiles are chosen for transfer to the uterus.</li> <li>• The validity of the results may be questioned due to bias in the ploidy comparison and determination process.</li> </ul>

### 3. Standard Sources of Preimplantation Embryo Genetic Information

Polar body (PB) biopsy was introduced as an approach to preimplantation testing with minimal risk of embryo damage, as its most prominent positive feature [12]. In addition, PB biopsy is applicable in countries with strict legislation against day-three biopsy or biopsy after syngamy. Nevertheless, the major drawback of this type of biopsy is the need for primary and secondary PBs; it is also relatively labor-intensive, only maternal chromosomes are examined, and it is not applicable in cases of abnormal paternal genetic contribution [13].

There are advantages and disadvantages to day-three preimplantation embryo biopsy as well [14]. The most positive aspects are that genetic information from both parents and the embryo is present, it can be performed with 6–10 cells, and blastomeres at this stage are assumed to be totipotent, representing the whole embryo, before differentiating to trophoctoderm and inner cell mass [15]. This type of biopsy was the prevalent biopsy method for almost two decades (1990–2010), in conjunction with FISH for aneuploidies and structural variations, and with PCR for monogenic disorders. Initially, two cells were biopsied, and later only one cell was biopsied, due to the observed and reported reduction in pregnancy rates, along with the measurable impact on the embryo [16].

The blastocyst biopsy of the trophoctoderm was introduced in 2011 and has remained the method of choice since then [17]. The more cells available (5–10 cells), the lesser the impact on the embryo's inner cell mass, rendering this type of biopsy safe, reliable, and effective, with no effect on the reproductive potential [18–20]. Nevertheless, in recent years, considerations have emerged, as the cells originate from the trophoctoderm only, meaning that the mosaic inner cell mass remains undiagnosed [21]. Furthermore, mosaicism in the trophoctoderm generates uncertainty over the safety of mosaic embryo transfers [22]. Blastocyst culture conditions per se produce fewer embryos to test, and biopsy conditions and operators create variability between labs concerning the percentages of normal embryos. Other drawbacks in blastocyst biopsy are the additional costs to standard IVF-ICSI, the need for extensive training, and the discarding of potentially viable embryos [23].

### 4. Origin of Embryonic DNA

Finding the source of the DNA is crucial for evaluating the blastocoel fluid (BF) and spent culture medium (SCM) DNA and how closely they reflect the growing embryo [24]. Extraembryonic DNA is thought to be released in small amounts as a result of the embryo's cell death mechanisms. DNA fragmentation is linked to certain processes, such as necrosis and apoptosis. Apoptosis may play a part in controlling the number of cells in the body or eliminating the presence of cells that are genetically aberrant or potentially detrimental to development [25]. It consequently manifests in human blastocysts that are in good condi-

tion. Neighboring cells can phagocytose apoptotic cells or shed them into the peritoneal space or blastocoel cavity [26]. Extraembryonic DNA may yield more significant information about the lineage that will ultimately produce the fetus than traditional trophoctoderm (TE) biopsies if apoptosis is mainly restricted to the inner cell mass (ICM). Bolton and colleagues (2016) used individual cell tracking in a chimeric preimplantation mouse model of mosaicism to find that aneuploid cells in the murine TE continue to proliferate abnormally, while those in the ICM undergo apoptosis to eliminate them [27].

According to Fabian et al. (2005), apoptotic cells can be ejected into the blastocoel cavity or perivitelline space, or they can be phagocytosed by nearby cells. When exposed and accessible internucleosomal linker DNA is cleaved by endonuclease, apoptosis occurs [28]. This results in oligomers and multiples that are approximately 180 bp in size [29]. According to Zhang et al. (2016), NGS analysis of pooled BF samples ( $n = 3$ ) indicated two native populations of DNA fragments: the first spanning 160–220 bp in length, and the second spanning 300–400 bp [30]. These findings are consistent with the theory that extraembryonic DNA originates from apoptotic cells [31]. Many mammals have been reported to experience waves of apoptosis in their ICMs; however, levels of both ICM and TE lineages in human IVF embryos seem to be comparable (7–8% of cells) [32,33].

Comprehending the release of DNA from the BF and SCM as a result of normal or pathological events is crucial. Which embryos—those with poor viability and morphology, which may have higher rates of cell death—have more DNA available in the BF/SCM? Those with good morphology have numerous cells. Regarding the relationship between successful extraembryonic DNA retrieval and embryo quality, research to date has produced contradictory findings [34].

Although Zhang and colleagues found no correlation between the amount of BF-DNA and embryo quality, other teams have found that blastocyst morphology and whole-genome amplification (WGA) efficiency are positively correlated, and that fully expanded day-5 blastocysts are more likely than less-developed embryos to yield detectable extraembryonic DNA [24,35]. Apoptosis marker caspase-3 activity and BF-DNA content were found to be positively correlated with each other, as well as with embryo quality, according to Rule and colleagues [36]. On the other hand, a cohort of embryos with an aneuploid trophoctoderm biopsy diagnosis (81% of 185 samples) had WGA rates (SurePlex, Illumina) that were significantly higher than those with a euploid diagnosis (45% of 71 samples) [35].

Furthermore, the group with unsuccessful BF amplification had higher clinical pregnancy rates (77% vs. 37%) when 53 paired embryos were used for IVF [37]. The authors conjectured that, in light of these findings, failed BF amplification could function as an extra selection factor to help prioritize embryos for transfer during in vitro fertilization. Moreover, it is possible that the embryo was harmed by the sampling processes, resulting in at least some of the DNA found in the blastocoel. When cells accidentally lyse during blastocentesis, their nuclear contents may leak into the BF and be aspirated [38]. This could happen if the TE layer is penetrated. Furthermore, it is not known whether extraembryonic DNA is always found outside of cells, or if it is occasionally found inside membrane-enclosed cells that are not part of the embryo [31].

Owing to the diameter of the ICSI pipette that is usually used to collect BF, it is not completely possible to avoid sampling of whole cells floating in the BF. According to studies conducted in 2015 and 2019 by Poli and by Battaglia and colleagues, respectively, the identification of proteins, extracellular vesicles, and micro-RNAs (miRNAs) in human BF suggests that the blastocoel may actively contribute to intercellular communication during embryo development and implantation [39,40]. Such extracellular vesicles might also include a fraction of extraembryonic DNA [41].

## 5. Alternative Sources of Preimplantation Embryo Genetic Material

The sources of genetic material competent for clinical preimplantation testing, apart from the TE, are mainly two extraembryonic sources: the BF, alternatively termed blastocoel cavity fluid (BCF), and the spent embryo culture medium (SCM), which both contain

embryonic DNA and other nucleic acids with value for prognostic embryo testing [42]. BCF fills the blastocoelic cavity synchronously to the formation of the blastocyst and is therefore valuable to aspirate and use as a cell-free sample. BCF aspiration is a minimally invasive approach targeting a volume of almost 4 nL, without any kind of cell extraction or cell damage [36]. The aspiration of the BCF has been suggested and implemented as a preparative step for blastocyst freezing, with validated clinical results, and is therefore appropriate for the preservation of blastocysts following the aspiration step [43]. SCM testing is the epitome of the non-invasive approach, as it simply requires the collection of the remaining medium when the blastocyst is removed to a new culture medium droplet [44]. Nevertheless, the collection of the SCM requires a change of culture medium, even if it is not required by the process implemented when using one-step culture media. This may also require an additional culture day for the collection of a richer nucleic acid medium [45].

## 6. Recommendation of Trophectoderm Biopsy Replacement

What can now be tested by the invasive PGT on the biopsied trophectoderm (TE) cells is a broad spectrum of genetic information residing in the blastocyst, such as numeric (including ploidy) and structural chromosome constitution, mosaicism, copy number variations (CNVs), single-nucleotide variants (SNVs), methylation, DNA fingerprinting, contamination, and mRNAs and microRNAs (including piRNA, snoRNA, etc.) (ESHRE data, Dagan Wells JUNO Genetics).

The contemporary gold standard, with TE biopsy and 24-chromosome testing, has proven to be efficient and safe in several RCTs, to reduce chromosomally abnormal pregnancies, and also to reduce the time to pregnancy and the overall treatment costs, with no impact on the so-called cumulative live birth rate (CLBR). In addition, TE biopsy has proven high reliability when applying strict criteria for mosaicism stratification [46–51].

Nevertheless, TE biopsy has some characteristic features that the community acknowledges as limitations to the broad and uncompromised use of PGT [17]. First, the training in biopsy is time-consuming, and the practice itself is delicate and time-consuming, requiring dedicated and expensive equipment to perform. There is also a risk of embryo damage and stress, as well as high variability in the results between operators and laboratories [52]. As TE biopsy broadly depends on the chance to extract cells from the unaffected part of the embryo in case of mosaicism, the probability of wrongly diagnosing an embryo is high, and the risk of discarding a healthy embryo is also high due to the opposite chances [53].

## 7. Non-Invasive Methods for Embryo Assessment

In the fields of in vitro fertilization (IVF) and assisted reproductive technology (ART), non-invasive techniques for evaluating embryos are becoming more and more significant. These techniques aspire to offer important insights on the health and development potential of embryos by employing non-invasive techniques like embryo biopsy [54]. First of all, time-lapse imaging requires taking ongoing pictures of the growing embryos at consistent intervals, without interfering with the environment of their culture. Embryologists can employ this technique to evaluate the regularity and timing of cell divisions, as well as to observe important developmental milestones. Embryos with the best development patterns can be identified owing to this continuous monitoring [55].

Metabolomic profiling is an additional non-invasive technique for evaluating embryos. Specifically, this approach examines the metabolic waste products generated by developing embryos [56]. Researchers can learn more about an embryo's metabolic activities by examining the chemical composition of the culture media used to cultivate it. Variations in metabolite levels may provide information about the health and viability of embryos. Furthermore, morphokinetic analysis falls under the same category as this approach, which assesses the morphological traits and the kinetics of embryonic development [57].

## 8. Automated Assessment

Automated techniques can assess features like cell symmetry, blastomere size, and timing of cell divisions by analyzing images or footage of growing embryos. Embryo quality may be indicated by deviations from typical developmental patterns [58].

Time-lapse systems provide digital images of embryos at frequent intervals, allowing embryologists to assess their quality without removing them from their culture environment [59]. Embryos can be transferred to the uterus at the cleavage or blastocyst stages. The blastocyst stage may increase the likelihood of selectively transferring viable embryos [60]. Correct identification of cell numbers is significant in determining the timing parameters for embryo quality evaluation [61].

Though advances in cell detection and tracking have been made, topological alterations and image noise continue to pose significant challenges to computer vision research. In medical imaging applications, variables in pertinent data influence decision-making [58]. Artificial intelligence has not received much attention as a tool for assessing human embryo development quality. A crucial method for upcoming human support technology is deep learning. Convolutional neural networks (CNNs) have enormous promise for use in medical diagnostics, imaging, and overall healthcare [62]. Time-lapse photos have been used to analyze embryos objectively and automatically through the development of a technique called STORK [63]. An extremely encouraging outcome was that the method could predict blastocyst quality with an area under the curve (AUC) [63]. According to a recent study, individual time-lapse imaging for mouse and human embryos up to the four-cell stage can be classified using a framework based on Inception-V3 CNNs [64].

For the assessment of embryos, oxygen consumption and culture conditions are two additional non-invasive methods. Information regarding the metabolic activity of growing embryos can be obtained by tracking their rate of oxygen consumption. Understanding of the energy needs and general health of embryos can be gained by using this non-invasive technique [65]. Regarding embryo culture conditions, optimizing the culture environment in which embryos develop is a critical aspect of non-invasive assessment. Factors such as temperature, pH, and nutrient concentrations can impact embryo quality [66]. Maintaining optimal culture conditions is essential for supporting healthy embryo development. It is important to note that time-lapse imaging and carefully regulated culture conditions are combined in a technique known as an EmbryoScope [67].

## 9. Benefits of Minimally Invasive and Non-Invasive PGT

Despite TE biopsy being the gold standard, its safety and reliability have been called into question by the emergence of PGT in embryo assessment [9]. Still, as long as they adhere to the established protocols, non-invasive and minimally invasive techniques are seen as unique innovations because they are less costly and easier to perform [68]. One of the main advantages is that less invasive biopsy stress is applied to the embryos due to less embryo manipulation [69]. Minimal intervention may be advantageous for the fetus's growth and well-being. The TE biopsy procedure requires appropriate handling as well as appropriate training [52].

This approach is expensive for both the couple undergoing IVF and the facility [70]. Deviating TE biopsy with alternative approaches may also result in higher rates of pregnancy, a broader range of patients receiving treatment, and lower costs. However, the use of embryo biopsy for genetic testing raises many moral and ethical questions, and this is something that needs to be acknowledged. Their beliefs and ideals support avoiding this process [4]. Clinical misdiagnosis and confusion surrounding PGT-A will persist even in the presence of the most precise genetic analysis. This is because there is proof that the present biopsy procedures may be hazardous, and because of the discrepancy between TE and inner cell mass (ICM) [71]. Nevertheless, to apply new technology, attempts have been made to make it less intrusive and to create complex algorithms. Promising methods include measuring DNA in spent embryonic culture medium and blastocoel fluid, measuring

mitochondrial DNA, metabolomics, measuring nutritional glucose intake, proteomics, and life-span microscopy [72].

When developing algorithms for the appropriate selection of embryos for transfer to humans in in vitro fertilization (IVF), for instance, the quick screening of glucose metabolism in the human embryo on day 4 and day 5 (morulae stage) may turn out to be beneficial [73]. The notion that male and female human embryos have different physiologies as a result of having two active X chromosomes and having a modified proteome for a limited period during preimplantation is further supported by the detected sex-related metabolic differences [74]. Hence, according to Gardner and colleagues, glucose consumption may be used as a marker to help identify competent embryos capable of supporting implantation, development, and live birth [73].

### 10. Blastocoel Fluid Aspiration

A typical human embryo develops into a fully developed blastocyst from 120 to 144 h after fertilization. The embryo typically has a fully formed blastocoel at this point, which is a cavity filled with fluid and enclosed in a layer of trophoctoderm (TE) cells. The inner cell mass (ICM), a collection of cells connected to the inner side of the TE layer and expanding into the cavity, is in contact with the blastocoelic fluid, which we will refer to as blastosol in this instance [39].

The cavity known as a blastocoel, which has an average capacity of 4–6 nL, begins to form on day 4 and fully develops between days 5 and 6. The genetic material is amplified to a great degree of variety, as suggested by the variations in amplification rates [39]. Significant differences in the outcomes between PGT-A and blastocoel are also observed. A related concern that emerges and can be addressed is related to the variations in the genetic coherence between trophoctoderm and blastocoel cells [11].

Embryonic proteins are discharged into the blastocoel, where they may accumulate. According to the study conducted by Watson and colleagues, there is strict regulation over the movement of molecules into and out of the enclosed blastocoel fluid [75]. The surrounding monolayer of TE cells forms a solid barrier separating the blastocoel from the external environment through tight connections. Due to the absence of impurities from the culture medium, the blastocoel can offer an extremely pure sample of embryo secretions. To acquire blastosol samples from viable embryos, a micromanipulation technique known as blastocentesis was developed. Embryonic DNA was collected using this methodology for preimplantation genetic screening before invasive biopsy techniques were developed (Figure 2) [76–78].

#### PROS AND CONS

Blastocoel fluid aspiration

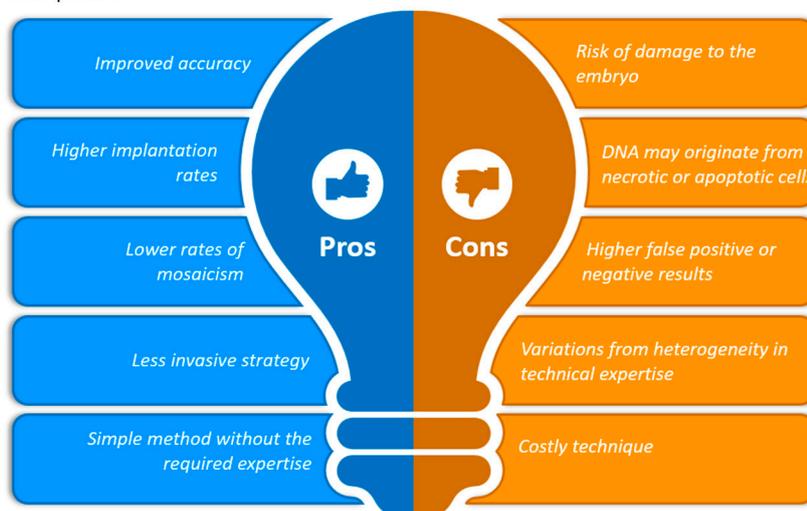


Figure 2. The benefits and drawbacks of blastocoel fluid aspiration.

## 11. Spent Embryo Culture Medium (SCM)

Research has concentrated on metabolites, proteins, interleukins, and microRNAs, and it has been investigated whether measuring specific chemicals produced could be useful in predicting the reproductive competence of embryos [79]. The wide range of substances that embryos leak into the medium—known as the secretome—have been the subject of multiple studies over the last ten years. Assessing the spent culture medium (SCM) of the embryo presents a viable substitute for the non-invasive genetic evaluation of preimplantation embryos.

Examining genetic information, it appears that SCM may occasionally contain more DNA than the blastocoel fluid (BF). In contrast to blastocentesis, which still involves some degree of embryo manipulation, this implies that SCM could provide a source of material for a completely non-invasive genetic testing technique [38]. There have been reports of the discovery of mitochondrial DNA (mtDNA) and genomic DNA (gDNA) in SCM as early as days 2–3 of development [80]. The amount of DNA has been seen to rise during embryo culture, indicating that a significant portion of the genetic material found in SCM has an embryonic origin, even though various media formulations may contain DNA contaminants, which is a reason for concern when thinking about genetic testing [81].

Nucleic acids are expected to be able to move from the preimplantation embryo into the medium with little resistance because, despite the zona pellucida protective glycoprotein barrier surrounding it, it has a high degree of flexibility, even the relatively large macromolecules [82]. Nevertheless, it is still unknown what processes lead to the release of embryonic DNA. The gDNA in the SCM is most likely extremely low in quantity, degraded, and/or the product of cell death processes, similar to the blastocoel, according to a comparison of the observed PCR amplification rates from SCM with those from cellular biopsies [69].

Even though apoptosis and the resulting DNA release seem to be typical aspects of blastocyst development, other mechanisms might be required to account for the embryonic DNA found in SCM at earlier stages. According to studies, cell death that occurs before compaction is related to fragmentation and embryo arrest, and healthy, normally developing human embryos do not exhibit any signs of apoptosis before the blastocyst stage [83,84]. Consequently, Stigliani and colleagues set out to examine the role of gDNA and mtDNA in day-3 SCM as a biomarker of embryo quality. It is interesting to note that, when comparing SCM samples from poor-to-average morphological grades to samples from high-quality embryos, the amounts of total double-stranded DNA (dsDNA) were higher. Furthermore, the dsDNA was primarily of mitochondrial origin, according to further qPCR for single-copy nuclear and mitochondrial genes. The number of copies of mtDNA in the medium was also significantly correlated with increasing maternal age and the degree of embryonic fragmentation [85].

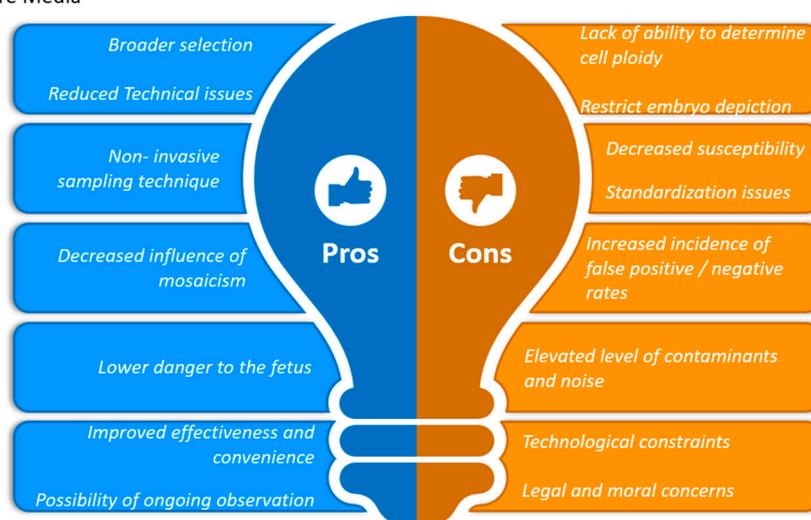
Based on these findings, it was believed that nuclear–cytoplasmic fragments, not apoptotic bodies, were the primary source of DNA in the SCM. Since a single-copy nuclear gene was employed for normalization, which is not ideal for quantifying mtDNA in samples with very low DNA concentrations (especially if the DNA is degraded), care must be taken when interpreting this data. In the study conducted by Hammond and colleagues, it was noted that cleavage-stage embryos with low or no fragmentation in the SCM included mtDNA and gDNA, after using single- and multi-copy gene quantitative PCR (qPCR) markers [86].

In addition, Stigliani's group subsequently demonstrated that implantation results, trophoctoderm quality, and subsequent blastulation rates were all favorably correlated with the mtDNA-to-gDNA ratio in day-3 SCM [85]. There is yet no confirmation of the degree to which the relative ratio of gDNA or mtDNA, or their total levels in the SCM, signal embryo viability. Certain results are in line with those of blastocoel. Initially, a significant fluctuation in the nucleic acid DNA content amplification rate is noted. Furthermore, there are differences in consistency between PGT-A and SCM. Overall, compared to blastocentesis, the outcomes of SCM were generally more in line with those of invasive biopsies [11].

Before being used in clinical practice, SCM presents with extra difficulties. Such obstacles include significant variances in analytical techniques, as well as sample contamination. The outcomes, however, are encouraging when certain changes are made to the existing techniques for embryo culture [87]. First, a modification should be made the day after the culture medium; that is, on day 4, when sequential media are utilized and appropriate for the SCM procedure to be applied, rather than on day 3 [88]. Additionally, it is advised to reduce the volume of the embryo culture medium, with a value range of 5 to 30 milliliters, since smaller volumes may be handled and processed more easily and do not require further procedures. Furthermore, blastocysts that are fully grown by day 5 can be obtained by prolonging the culture period to day 6 or even 7 [89]. Finally, the advent of single-stage media that can maintain embryo development throughout the blastocyst stage may mean that numerous changes to the culture media are not required (Figure 3) [23].

### PROS AND CONS

Spent Culture Media



**Figure 3.** Advantages and disadvantages of spent culture media (SCM) concerning non-invasive PGT.

## 12. Diagnostic Performance of Blastocyst Culture Media in Non-Invasive PGT

Employing trophoctoderm (TE) biopsy in conjunction with preimplantation genetic testing (PGT) has proven beneficial for millions of couples in conceiving healthy offspring by identifying embryos that have chromosomal and/or genetic abnormalities [90]. Nonetheless, numerous issues persist concerning the precision of diagnosis and the security of this method. A five-to-ten TE cell biopsy still cannot be considered a perfect reflection of the inner cell mass (ICM)'s true chromosomal state. Furthermore, chromosomal mosaicism in blastocyst-stage embryos may impair the diagnostic precision of a single TE biopsy [91,92]. A single sample containing two or more cell lines with distinct genotypes is known as chromosomal mosaicism [93].

In human preimplantation embryos, this has been widely detected, in contrast to later placental samples. Currently, several studies have shown that the rising number of chromosomal mosaicism reports may be associated with the greater sensitivity of the TE biopsy itself [50,93,94], as well as with the NGS platform [94,95]. A healthy live birth could nonetheless occur from the transfer of embryos identified as "abnormal" by TE biopsy in PGT for aneuploidy (PGT-A), indicating the limitations of TE biopsy's diagnostic accuracy [46]. Furthermore, there is always a chance that the viability of embryos will be compromised by invasive biopsy procedures [5,96]. In this case, investigations are being carried out to confirm whether utilizing cell-free DNA (cfDNA) for non-invasive preimplantation genetic testing for aneuploidy (niPGT-A) could be promoted as a viable method for aneuploidy screening.

A new era of possibilities for niPGT-A was opened by Palini and colleagues, who successfully amplified cell-free DNA samples in blastocoel fluid (BF) to identify embryos with X-linked illness [97]. It was subsequently discovered that the extracellular cfDNA in discarded culture media included a greater amount of genetic material related to medicine than that in BF [98]. Even though the use of cfDNA in niPGT has been encouraged, its initial source and makeup remain unknown; it may have been contaminated with a combination of maternal cells and embryonic DNA [99]. Furthermore, there is ongoing debate regarding the consistency of ICM and cfDNA from BF or spent culture media [69,100–102].

Previous studies were conducted to compare the diagnostic efficacy of niPGT-A and TE biopsy, or to assess the concordance between niPGT-A and TE biopsy PGT-A, using the remaining portion of the embryo or the entire embryo as the standard reference [46,100,103–105]. Nonetheless, a growing body of research has shown that mosaicism may cause the TE biopsy results and the remainder of the embryo mixed with ICM and TE to inaccurately represent the genetic state of the blastocyst-stage embryo [46,106–108].

Comparing BCM with many embryonic biopsy sites, especially the ICM samples, would be a more cautious and advisable method to confirm the diagnostic efficacy of niPGT-A [109]. The diagnostic effectiveness of niPGT-A has never been thoroughly assessed before, as far as we are aware. It compares the concordance of ICM, BCM, and TE (i.e., initial TE and TE re-biopsy) samples. Chen et al.'s study revealed that three partially "mosaic" embryos identified by initial TE biopsies were euploid upon analyzing TE re-biopsies, BCM, and ICM samples. This implies that TE biopsy might not be a trustworthy marker of ICM's true chromosomal condition.

The self-correcting process in mosaic embryos [110], which allows mosaic cells in the cleavage-stage embryo to be incorporated into TE cells but not ICM cells, may help to explain this. The actual manipulation of the biopsy could be another reason. According to reports, laser biopsy manipulation can cause mosaicism, cell injury, and the loss of cellular DNA [93]. This can therefore cause bias in the development of libraries. Furthermore, higher-resolution NGS-based PGT-A shows improved sensitivity to detect lower-level mosaicism [111,112], in contrast to earlier technologies like array comparative genomic hybridization (aCGH) [113].

An additional issue in diagnosing lower-level mosaicism arises from the fact that euploid/aneuploid mosaic embryos are regarded as abnormal in between euploid and abnormal embryos. Carefully determining the threshold for mosaicism is necessary to classify the embryos as euploid, aneuploid, or mosaic. According to the Preimplantation Genetic Diagnosis International Society [93], the mosaic spectrum runs from 20% (much-reduced risk) to 80% (greater risk), and this information should be taken into account and reported. Various laboratories may have different cutoff values for reporting mosaic levels because of varying degrees of ability to detect mosaicism. This could potentially impact the precision of TE biopsies and wasted BCM.

Based on NGS results, Maxwell and colleagues found that 31.6% of the embryos that were miscarried but had previously been identified as euploid were found to be mosaic using the 20% to 80% mosaic criterion [111]. A different study showed that similar pregnancy outcomes to euploid embryos were associated with mosaic embryos that had fewer defective cells (<50%) [114]. In their PGT laboratory, the lower cutoff value for mosaic was 40%, while the upper value was 70%, to reduce the false positive rate (FPR) and false negative rate (FNR). Three "mosaic" embryos in Chen and colleagues' investigation had mosaicism values of 40% (monosomy 5), 30% (monosomy X, trisomy 18 and 21), and 50% (monosomy X). However, the uniform cupidity of the samples from the TE re-biopsies, ICM, and BCM shown by the test findings suggested that the mosaics in the first TE biopsies were related to false positives rather than the incorrect threshold for mosaicism.

It should be highlighted that embryos with the first TE biopsy diagnosed with chromosomal mosaicism are often discarded. Nonetheless, it has been demonstrated that PGT-A embryos transferred with TE biopsy diagnoses of "aneuploidy" might nevertheless produce live, healthy children, demonstrating the limitations of TE biopsy's diagnostic

precision [106,113,115,116]. Alternatively, BCM sampling, being a non-invasive procedure, may reduce the bias previously described, making it more dependable for predicting the ICM karyotypes, and potentially preventing the loss of normal embryos [106,113,115].

Furthermore, niPGT-A with a BCM sample may attain diagnostic efficacy comparable to that of TE biopsy, and in the three aforementioned partial “mosaic” cases it proved to be congruent with the ICM findings. Additionally, niPGT-A with BCM in conjunction with an initial TE biopsy could result in a more promising diagnostic accuracy. For instance, the highest priority for transfer is given to embryos that show the same euploid results from both the TE biopsy and niPGT-A [90]. Similarly, the lowest priority for transfer is given to embryos that show euploid results from niPGT-A and mosaic results from the first TE biopsy, rather than them being disposed of directly. More research is required on the combination of TE biopsy and niPGT-A, and in clinical applications the test costs should be taken into account.

The clinical applicability of niPGT-A is contingent upon its efficacy in amplifying sufficient cfDNA, in addition to its diagnostic capabilities. Both TE and ICM may theoretically leak cfDNA into the culture material, but TE comes into direct contact with the medium, while ICM does not [109]. Through artificial blastocyst collapse by laser, cfDNA was released from BF into the spent BCM, increasing the concentration of cfDNA with the least amount of damage. The 26 donated embryos used in this investigation all produced effective DNA amplification, which was higher than the efficiency shown in other studies [69,97,117]. Furthermore, it is reasonable to assume that this manipulation, when used in sample collection in niPGT, may not cause much damage to the embryos, since it artificially collapses the blastocoel cavity in enlarged blastocysts, which may enhance the vitrification result [69,97,117,118].

### 13. Diagnostic Performance of BF and SCM as PGT DNA Sources in Clinical Settings

The genotypes of the embryos produced during PGT-M cycles were compared among the various specimens in the first section of this investigation. The spent culture medium collected from the blastocyst-stage (SBM) samples yielded a considerably higher diagnosis rate than the BF samples, albeit inferior to that of the TE samples. Even though SBM can be diagnosed, 10.1% of the loci studied had a high detection of artifacts or allele drop-in (ADI). The inclusion of foreign DNA in the culture media is one of the possible causes of this noteworthy rate of detectable nonembryonic DNA material [24]. Indeed, many brands of new, unused media have been observed to contain trace levels of DNA [86,99]. The most likely way that these contaminants are added to the media is by adding non-pure DNA-binding proteins, including albumin. PCR is a useful tool for detecting this kind of DNA contamination in culture media samples, even though it is rare and potentially innocuous during traditional IVF procedures.

Interestingly, in both the BF and SBM groups, the allele dropout (ADO) rates for paternal alleles were statistically considerably greater than those for maternal ones. This initial finding indicated that mother DNA was more represented in DNA than paternal DNA [24]. Accordingly, it is likely that genetic material from the polar bodies or cumulus complex is still present in the culture system and is retrieved using embryonic DNA for analysis. Further support for this notion came from the discovery of the mutant allele of maternal origin in the SBM samples, even if the corresponding TE was homozygous for the wild-type gene. These findings offered strong proof of the significant amount of maternal DNA present in SBM samples (Table 2) [24].

While some recent research has recommended the clinical use of SBM for aneuploidy testing [105,119] or PGT-M [120], these findings show that more research is necessary before this diagnostic strategy can be taken into consideration in clinical settings. To increase the proportion of embryonic DNA and stop nonembryonic DNA carryover, different approaches could be used. We suggest pretreatment DNA depletion in culture media, total oocyte elimination, and temporal standardization (with regard to blastocyst growth) for BF and SBM collection, among other things. A modified variation of Chan et al.’s second-

generation non-invasive fetal DNA analysis could be used to amplify embryo-specific DNA [30]. A different, efficient method of obtaining embryonic DNA for genetic testing selectively could be the isolation and focused examination of exosomes released by the blastocyst in the culture medium [121].

**Table 2.** An overview of the main findings concerning the systematic reviews of non-invasive PGT-A.

Cell-Free DNA SCM	Cell-Free DNA BF
The ploidy of the embryo was unaffected by cfDNA.	Cells separate into TE and ICM on the 4th day of blastocyst development. Blastocoel forms withing the blastocyst during cavitation.
An estimated 86–94% of instances involve contamination with maternal genetic material.	Through blastocentesis → BF aspirated. About 0.01 µL of BF can be isolated.
The seed embryo’s embryonic DNA content ranged from 0% to 100%. Not every embryo’s embryonic genome may be evenly represented in the seed embryo.	Only 34.8% of BF samples were able to produce a signal suitable for embryo karyotyping. This outcome might have been caused by the fact that fresh blastocysts were used for the blastocentesis.

According to the PGT-A data from the study by Capalbo and colleagues, only 34.8% of the BF samples were able to produce a signal suitable for embryo karyotyping [24]. Before the embryos were cryopreserved or had a biopsy (under experimental circumstances), BFs were obtained in sizable investigations of the usage of BF for PGT. On the other hand, their study replicated a real PGT cycle therapy (under clinical circumstances) by collecting blastocoel samples from fresh, untreated blastocysts [24]. When compared to the results that we obtained under clinical conditions, the amplification rates under experimental conditions were higher (62%, 82%, and 96%) [35,102]. Perhaps increased DNA availability in the cavity as a result of cell lysis induced by cryopreservation or micromanipulation led to the higher DNA amplification rates seen in these investigations [122].

According to published data from Tobler’s and Werner’s groups, only a small percentage (37.5%) of the amplified samples in our dataset produced results consistent with those obtained from TE biopsies (53% and 72% of amplified samples matched the initial embryo diagnosis, respectively) [102,123]. Only a small percentage of the incongruous instances identified can be associated with the application of various methods for aneuploidy evaluation, as qPCR and VeriSeq PGT-A techniques generated concordant results for uniform aneuploidies when examined on cell line specimens. It is extremely difficult to identify the biological basis of such discordances, because functional research on the biological mechanisms of embryonic DNA release in the extracellular environment is inadequate [24]. Membrane-encapsulated DNA may come from DNA-containing pieces from apoptotic cells, from selective degeneration of aberrant cells in mosaic diploid/aneuploid embryos, or from improper (or corrected) chromosome segregation mechanisms during cell division [102].

The analytical sample may be seriously contaminated by this kind of non-representative DNA, seriously endangering the diagnostic sample’s accuracy [124]. However, at this time, there is not enough evidence to confirm that human preimplantation embryos have aneuploidy correction mechanisms. Enigmatically, fluidic samples may prove to be even more accurate in forecasting the genetic makeup of the blastocyst than TE biopsies if all embryonic cells release their DNA uniformly [38].

Through the comparison of vitrified and/or warmed embryos and culture media ploidy results, Xu and colleagues stated that a detailed examination of non-invasive chromosomal screening (NICS) revealed that the test was confirmed under experimental settings [105]. Of the 42 pairings of samples, 38 showed diagnostic consistency at the embryo stage, while only 62% of the samples had full karyotype concordance. Additionally, by withholding genotyping data, the investigators were unable to determine whether nonembryonic DNA contamination was present in the samples, or where they originated. Moreover, given the absence of a control group in the research, the six live births that

seven couples who underwent NICS-based embryo selection were able to achieve did not constitute sufficient proof to back up the therapeutic efficacy of the NICS intervention [105].

#### 14. Mosaicism

The existence of multiple genotypically different cell populations within a single zygote is known as mosaicism. It is believed that, during post-zygotic cell division, mosaic cellular populations result from post-zygotic mitotic mistakes [125]. Mosaicism in PGT-A is characterized by a 20% to 80% mixture of euploid and aneuploid DNA; euploid DNA is found in less than 20% of the sample, while aneuploid DNA is found in more than 80% of the sample. Although 5% is the stated incidence of mosaic embryos, some researchers have used PGT-A to find rates as high as 20% to 30%. Embryos in the cleavage stage (30–70%) are more likely to exhibit mosaicism than those in the blastocyst stage (5–15%) [72].

Depending on the timing of mitotic mistakes in the blastocyst stage, and on the cell lineage, there are four types of mosaicism: A “total mosaic” embryo has both euploid and aneuploid cells in both the TE and the inner cell mass (ICM). The embryo is classified as “ICM mosaic” if the mosaic population is solely ICM, and as “TE mosaic” if it is purely TE. Finally, an embryo is considered to be “ICM/TE mosaic” if every cell in the ICM is euploid and every cell in the TE is aneuploid, or vice versa [126].

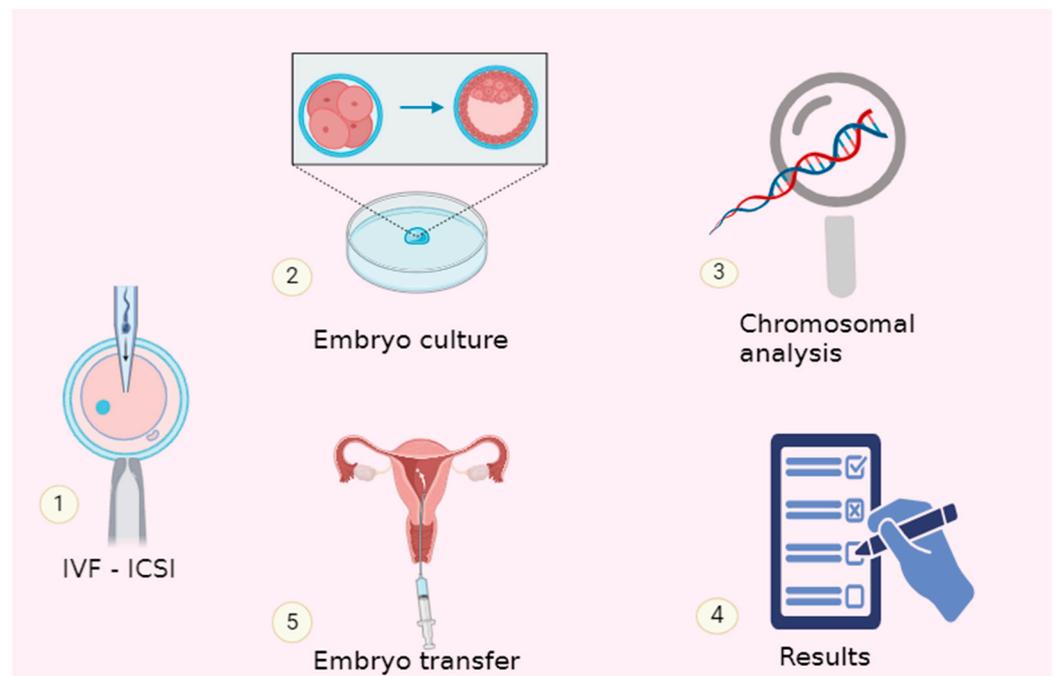
Euploidization in mosaic embryos may cause DNA to be liberated from aneuploid cells, increasing the likelihood of a false positive aneuploidy diagnosis. As euploid cells remain in the ICM, the DNA expelled into the BF would be aneuploid. According to several studies, it is known that certain mosaic embryos can implant and produce euploid offspring, indicating the possibility of mechanisms for the removal of defective cells [113,116,127].

Aneuploidy and chromosomal mosaicism are known to result from aberrant cell division in embryos. A study based on the theory that euploid and aneuploid cells would not contribute equally to the DNA contained in the culture media revealed that such embryos could occasionally deliver healthy blastocysts [128]. Despite the euploid nature of the biopsies taken from these developing embryos, it was shown that the excluded cells in these chromosomally normal blastocysts were aneuploid, and that compaction often resulted in the exclusion of some cells. If apoptosis or other mechanisms of cell death or exclusion can be used to repair mosaic embryos, more studies on human embryos are required to validate this conclusion. Based on published research with limited data on extraembryonic DNA, BF and SCM samples may have more aneuploid events than paired TE biopsies [35,129]. If confirmed, this would cast doubt on the idea underlying the PGT-A study of extraembryonic DNA or shed light on the fraction of aneuploidies undiagnosed by PGT-A.

Mosaic human embryos refer to embryos that have a mixture of normal and abnormal cells, often due to genetic mutations or chromosomal abnormalities. These embryos can result from errors in cell division during early embryonic development. The concept of mosaic embryos has significant implications in the field of reproductive medicine and assisted reproductive technology, particularly in the context of in vitro fertilization (IVF) and preimplantation genetic testing (PGT) (Figure 4) [130].

In preimplantation diagnostic research, the detection of mosaicism carries significant implications for genetic counseling. During oogenesis, as opposed to spermatogenesis, nondisjunction is more frequent. In cases where the offspring has a non-mosaic trisomy for a particular chromosome, gonadal mosaicism should be considered, as the recurring risk of mosaicism is unclear. The presence of germline mosaicism provides a basic explanation for the recurrence of uncommon mutations within a single family, and it may aid in the familial aggregation of affected people. Genetic counseling is dependent on the type of mosaicism, as each chromosome that is afflicted presents with unique clinical signs [125].

In monosomies, mosaicism has an impact on survival rates. While monosomies in humans are lethal, mosaic monosomies, which occur when more than 70% of cells are normal and the remaining monosomes persist, may not result in death after delivery.



**Figure 4.** The chromosomal composition of the embryo can be ascertained without an embryo biopsy through non-invasive preimplantation genetic testing, or niPGT-A.

Malignant conditions are caused by mosaicism. As people age, there is a noticeable, gradual loss of certain human genes, which causes tissue to become mosaic. Malignant tissue (such as neurofibromatosis) may also develop from the mosaic tissue.

## 15. Conclusions

In clinical practice, niPGT may lower expenditures per couple and increase the number of couples seeking PGT if it is validated and successfully applied. Furthermore, it might result in important developments for IVF/ICSI procedures in the future. In the first instance, it will enable and facilitate genetic analysis in embryos that, according to existing standards, are not appropriate for biopsy. Furthermore, because of technological advancements, this procedure can be used without running the risk of harming the fetus. In terms of where the genetic material derives from, it seems that blastocoel fluid aspiration (blastocentesis) is not as efficient a source of cfDNA as SCM (spent culture medium). Undoubtedly, investigations that significantly deviate from mainstream embryological procedures seem to obtain the best niPGT results.

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