Chapter

Non-Invasive Chromosome Screening for Embryo Preimplantation Using Cell-Free DNA

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Abstract

Preimplantation genetic testing (PGT) is widely adopted to select embryos with normal ploidy but requires invasive embryo biopsy procedures. Therefore, non-invasive PGT (niPGT) detection of cell-free DNA (cfDNA) in blastocyst culture medium has gradually become a hot area in the field of assisted reproduction. This chapter will systematically summarize how researchers use embryonic cfDNA to conduct niPGT detection worldwide. It will also thoroughly review the factors that affect the accuracy of the test and its underlying issues, as well as prospective applications. We hope to provide a useful reference for the standardized operation of non-invasive PGT that can be widely applied in clinical practice.

Keywords: niPGT, Spent Culture Media, cfDNA, Aneuploid

1. Introduction

In vitro fertilization and embryo transfer (IVF-ET) is an effective method for the treatment of infertility, yet it still has a relatively low success rate [1]. Furthermore, the multiple pregnancy rate remains high as a result of multiple embryos being transferred, which can increase the chances of adverse pregnancy outcomes and affect the health of both the mother and children [2]. Elective single embryo transfer (eSET) is the most effective way to reduce the rate of multiple pregnancies and is increasingly used worldwide [3]. Nonetheless, the success rate of single embryo transplantation is not satisfactory, mainly due to the lack of a systematic approach for evaluating the conceivability of embryonic development. Presently, embryonic morphological assessment is still the most commonly used method, but this method has many problems due to the lack of quantifiable indicators and its susceptibility to subjective factors of laboratory embryologist [4–6].

Studies have shown that there is an approximately 40%–60% risk of chromosomal aneuploidy even before embryo implantation [7–9]. PGT-A can be used to identify embryos with chromosomal aneuploidy, thereby improving clinical outcomes in IVF patients [10–13]. Nonetheless, PGT-A relies on embryo biopsy, and its

invasive biopsy approaches may increase its technical limitations for the following reasons: (1) it may have an influence on the quality and the level of development of the embryo [14]; (2) it may increase the chance of abnormal epigenetic modifications [15, 16]; (3) it has high requirements on the environment and operating instruments, and there is a potential risk of sampling failure; and (4) the accuracy of test results may be affected by mosaicism. Consequently, it is necessary to establish an optimal selection procedure in line with the morphological assessment for embryos that truly reflects the chromosome ploidy status of the embryo, avoids invasive operations, and does not require expensive equipment. Recently, several studies have found the presence of cfDNA in the culture medium and blastocyst fluid of embryos [17–21]. Noninvasive PGT-A detection through cfDNA has become a growing niche in the field of assisted reproduction. In this chapter, we will comprehensively review the advancements and attempts at noninvasive PGT-A using foetal cfDNA and discuss the pros and cons as well as insights into its clinical applications in IVF-ET.

2. cfDNA research based on Spent culture media (SCM)

2.1 The discovery of cfDNA in SCM

Stigliani et al. [18] first demonstrated the presence of cfDNA in SCM in 2013, where genomic DNA (gDNA) was found in 63% (205/326) of the 326 SCMs collected, ranging from 41 pg to 1.8 ng. Subsequently, Hammond et al. [19] also detected mitochondrial DNA (mtDNA) and gDNA in SCMs. In addition, Stigliani et al. [22] reported that the ratio of mtDNA to gDNA in the SCM of D3 was positively correlated with the formation rate of blastocysts. These results suggest that SCM can serve as a sampling source for early embryonic DNA, laying the foundation for the development of the non-invasive method of PGT-A.

2.2 Application of SCM in PGT-A

In recent years, an increasing number of studies have been conducted to evaluate the feasibility of SCM-based non-invasive PGT-A approaches, and some of the results are encouraging. Many studies have found that PGT-A by trophectoderm (TE) biopsy or whole embryo of SCM has a high consistency rate (as shown in **Table 1**. Pro). Nevertheless, other research groups have reported relatively low consistency rates when compared with TE or whole embryo results (see **Table 1**. Con). These contradictory results may be related to factors such as the difference in SCM sampling methods and sampling time used in the studies, embryo treatment, and the definition of consistency. By improving the detection method, non-invasive PGT-A using SCM is expected to meet the requirements of clinical diagnosis.

2.3 Factors affecting the accuracy of non-invasive PGT-A

When comparing the ploidy consistency of cfDNA in SCM, all studies have reported a high success rate of cfDNA amplification and detection, ranging from 77.3% to 100% (as shown in **Figure 1**). However, its consistency to the conventional PGT-A results fluctuated widely, ranging from 33% to 100% [23, 26, 27, 39] (as shown in **Figure 2**). Hence, it is critical to recognise the factors influencing accuracy.

Study	Fertilization method	Media system	Frozen: yes or no	Timing of collection and embryo manipulation	Volume for WGA	Amplification method	WGA products detection%(n)	DNA analysis	Concordance rate, %(n)
PRO									
Kuznyetsov et al. [23]	ICSI	Single medium	Yes	D5/D6 embryo (**freeze-thaw**): The thawed blastocysts were cultured in 25μL medium for 24h; Laser collapse to mix BF and SCM	-	SurePlex	100%(28/28)	NGS	96.4% vs WB (27/28) 87.5% vs TE (21/24)
Huang et al. [24]	ICSI	Single medium	Yes	D5/D6 embryo (**freeze-thaw**): The thawed blastocysts were cultured for 24h in 15μL medium, then the medium was collected	3.5μL	MALBAC	92.3%(48/52)	NGS	93.8% vs WB (45/48)
Jiao et al. [25]	ICSI	-	Yes	D5/D6 embryo (**freeze-thaw**): The thawed blastocysts were cultured in 12μL medium for 15h; Laser collapse to mix BF and SCM	10 μL	Improved MALBAC (MICS-Inst)	100%(41/41)	NGS	90.48% vs WB (19/21)
Li et al. [26]	ICSI	-	Yes	D5/D6 embryo (**freeze-thaw**): The thawed blastocysts were cultured in 15μL medium for 14∼18h; Laser collapse to mix BF and SCM	10 μL	Improved MALBAC (MICS-Inst)	97.6% (40/41)	NGS	87.2% vs WB (34/39)
Chen et al. [27]	ICSI	Sequential medium	No	D3-D5/D6 (Fresh): On day 3, each embryo was washed and moved to an individual 30-μL drop and cultured to blastocyst stage	20∼25 μL	MALBAC	100% (256/256)	NGS	78.1% vs WB (200/256)
Kuznyetsov et al. [23]	ICSI	Sequential medium	No	D4 ∼ D5/D6 (**Fresh**): On day 4, each embryo was washed and moved to an individual 10-μL drop and cultured to blastocyst stage, followed by laser collapse to mix BF and SCM	-	SurePlex	100%(19/19)	NGS	100% vs TE (19/19)

Study	Fertilization method	Media system	Frozen: yes or no	Timing of collection and embryo manipulation	Volume for WGA	Amplification method	WGA products detection %(n)	DNA analysis	Concordance rate, %(n)
Rubio et al. [28]	ICSI	Single medium	No	D4∼ D6/D7 (**Fresh**): On day 4, each embryo was washed and moved to an individual 10-μL drop and cultured to blastocyst stage(D6/7)	8-10μL	IonReproseq PGS Kit	94.8%(109/115)	NGS	84% VS TE (68/81)
				D4 ∼ D5 (**Fresh**): On day 4, each embryo was washed and moved to an individual 10-μL drop and cultured to blastocyst stage(D5)					63% VS TE (17/27)
Rubio et al. [29]	ICSI or IVF	Sequential medium or Single medium	No	D4 ∼ D6/D7 (**Fresh**): On day 4, each embryo was washed and moved to an individual 10-μL drop for at least 40 hours in culture.	10 μL	IonReproseq PGS Kit	92%(1197/1301)	NGS	78.2%(866/1108)
Lledo et al. [30]	ICSI	Single medium	No	D3-D5 (**Fresh**): On day 3, each embryo was washed and moved to an individual 20-μL drop and cultured to blastocyst stage(D5)	7.5μL	NICS-Inst	92.4%(85/92)	NGS	60.9% vs TE (28/46)
				D3-D6 (**Fresh**): On day 3, each embryo was washed and moved to an individual 20-μL drop and cultured to blastocyst stage(D6)					92.0% vs TE (34/37)
				D3-D5 (**Fresh**): On day 3, each embryo was washed and moved to an individual 20-μL drop and cultured to blastocyst stage(D5)	7.5μL	Sureplex	92.4%(85/92)	NGS	60.9% vs TE (28/46)
				D3-D6 (**Fresh**): On day 3, each embryo was washed and moved to an individual 20-μL drop and cultured to blastocyst stage(D6)					86.5%vsTE (32/37)

Study	Fertilization method	Media system	Frozen: yes or no	Timing of collection and embryo manipulation	Volume for WGA	Amplification method	WGA products detection%(n)	DNA analysis	Concordance rate, %(n)
Xu et al. [31]	ICSI	Sequential medium	Yes	D3 (**freeze-thaw**) ~D5: Warmed D3 embryos were placed in 30-μL droplets and cultured to blastocyst stage(D5)	5 to 20 μL	MALBAC	100%(42/42)	NGS	85.7% vs TE (36/42)
CON									
Hanson et al. [32]	ICSI	Sequential medium	No	D3/4-D5/6/7 (**Fresh**): On day 3, each embryo was washed and moved to an individual 30-μL drop and cultured to blastocyst stage, an additional media changeover occurred on day 4 for some samples.	-	NICS-Inst	62.7% (104/166)	NGS	59.6% vs TE (62/104)
Yin et al. [33]	ICSI	Sequential medium	No	D5/D6 embryo **(freeze-thaw)**: The thawed blastocysts were cultured in 25μL medium for 24h;	- (Collected 20-25 μL)	NICS-Inst	78.7% (59/75)	NGS	32.2% vs WB (19/59)
Yeung et al. [34]	ICSI	Sequential medium	No	D3-D5/D6 (**Fresh**): On day 3, each embryo was washed and moved to an individual 30-μL drop and cultured to blastocyst stage	3μL (Collected 20 μL)	SurePLEX	77.3%(116/168)	NGS	62.1% VS TE (72/116)
Li et al. [35]	ICSI	Sequential medium	No	D3-D5(**Fresh**): On day 3, each embryo was washed and moved to an individual 30-μL drop and cultured to blastocyst stage, followed by laser collapse to mix BF and SCM	NA (Collected 25 μL)	MALBAC	97.5%(39/40)	NGS	50% VS WB (19/38) 44.7% vs TE (17/38)
Vera-Rodriguez et al. [41]	ICSI	Sequential medium	No	D3-D5(**Fresh**): On day 3, each embryo was washed and moved to an individual 25-μL drop and cultured to blastocyst stage	20μL	SurePLEX	91.1%(51/56)	NGS	33.0% vs TE (17/51)
Feichtinger et al. [36]	ICSI	Single medium	No	D1-D5/6(**Fresh**): Embryos were cultured in single 25-μL droplets using a single step medium from fertilization until day 5/6.	5 μL	Sureplex	81.8%(18/22)	aCGH	72.2% vs PB (13/18)

Study	Fertilization method	Media system	Frozen: yes or no	Timing of collection and embryo manipulation	Volume for WGA	Amplification method	WGA products detection·%(n)	DNA analysis	Concordance rate, %(n)
Ho et al. [37]	ICSI	Single medium	Yes	D1 **(freeze-thaw)** ~D3: Warmed 2PNs embryos were cultured in 25-μL medium until D3	5 μL	Picoplex	39%(16/41)	NGS	56.3% vs WB (9/16)
				D1 **(freeze-thaw)** ~D5: Warmed 2PNs embryos were cultured in 25-μL medium until D5	5 μL		80.4%(33/41)		65% vs TE (26/40)
Liu et al. [38]	ICSI	Single medium	No	D1-D5(**Fresh**): Embryos were cultured in single 30-μL droplets using a single step medium from fertilization until day 5/6.	30 μL	MALBAC	90.90% (80/88)	NGS	64.5% VS TE (20/31)
Shamonki et al. [39]	ICSI	Sequential medium	No	D3-D5/D6(**Fresh**): On day 3, each embryo was washed and moved to an individual 15-μL drop and cultured to blastocyst stage	-	Repli-G	96.5%(55/57)	-	-
Galluzi et al. [40]	ICSI	Sequential medium	No	D1-D3(**Fresh**): Embryos were cultured in single 10-μL droplets from fertilization until day D3.	2.5μL	PicoPLEX	93.7%(30/32)	qPCR	
				D3-D5/6(**Fresh**): On day 3, each embryo was washed and moved to an individual 10-μL drop and cultured to blastocyst stage	2.5μL		94.4%(51/54)		
Stigliani et al. [18]	ICSI	Sequential medium	No	D1-D2/D3(**Fresh**): SCM were collected after embryo transferor cryopreservation on Day 2 or Day 3	-	GenomePlex	63%(205/326)	qPCR	

Table 1.
Concordance rates between niPGT-A based on SCM and TE or WB.

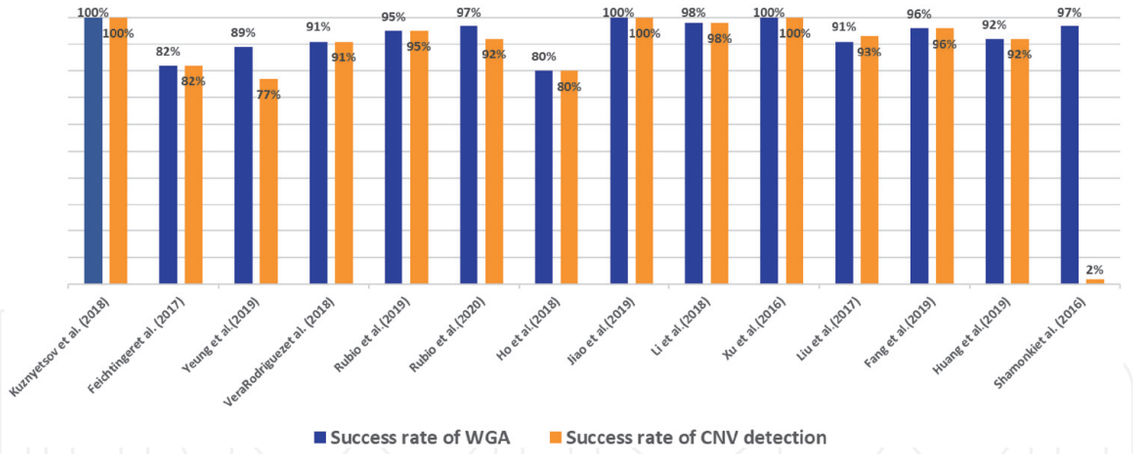

Figure 1.
Success rates of WGA amplification and CNV detection reported in different niPGT studies.

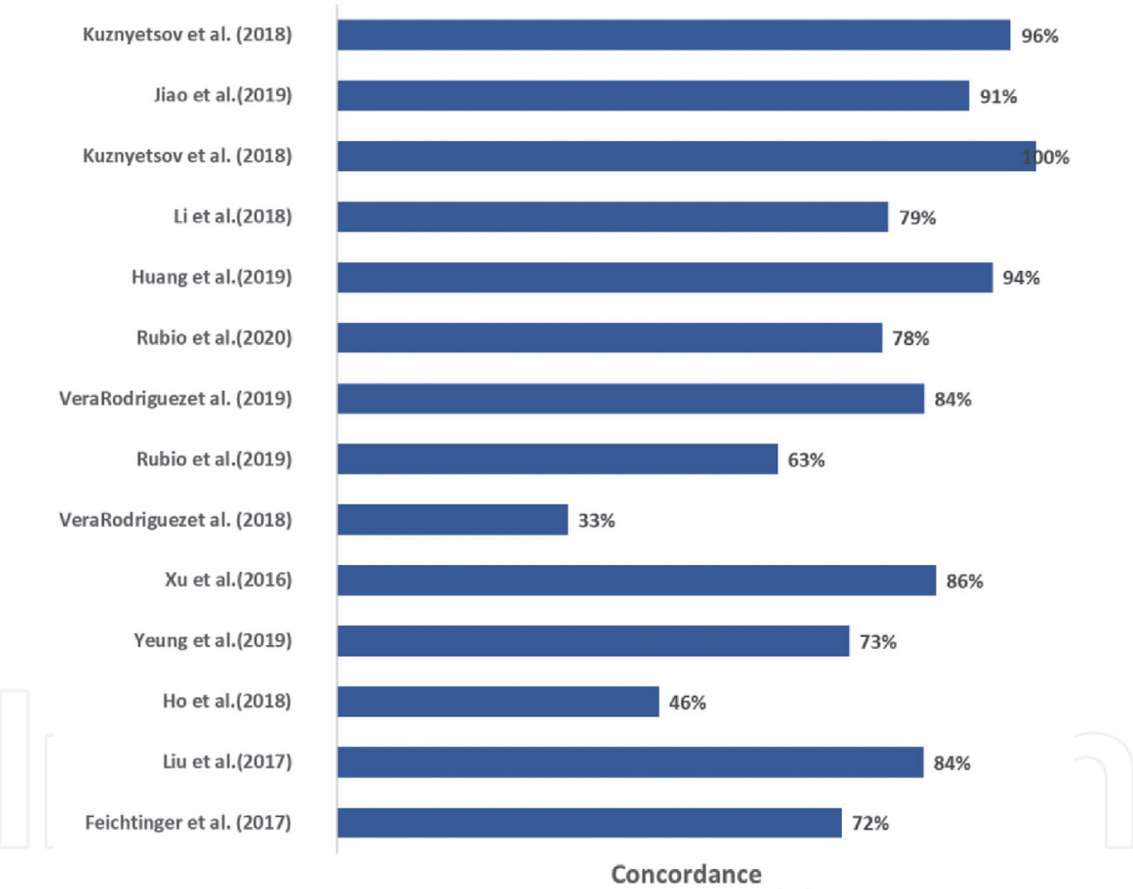

Figure 2.
CNV concordance rate reported in studies comparing SCM with TE or whole embryo samples.

2.3.1 Influence of sampling time on accuracy

At present, there are two common types of commercial embryo culture systems: sequential culture and single culture systems. However, the quality of DNA in SCM degrades as a result of cfDNA degradation over time. Therefore, the time and opportunity for cfDNA degradation could be minimized via media changes. Additionally, the maternal DNA contamination introduced by residual cumulus cells could be reduced or eliminated by media change to a certain extent. The results of a few studies using a single culture system [36–38] and sequential culture medium [31, 39, 41] are listed in **Table 1**. Importantly, Rubio et al. showed no significant

difference in the influence of different culture systems on consistency when performing the same sampling process [29] (as shown in **Figure 3**).

As the fertilized egg develops, DNA is constantly released into the SCM, which is a dynamic process. The test results of SCM collected at different time points during embryo culture may vary. Using a single medium, Ho et al. [37] achieved detection success rates of 39% (16/41) and 80.4% (33/41) when SCMs were collected on D3 and D5, respectively. When comparing the SCM collected on D3-D5 and D4-D5, Lane et al. [43] found that the accuracy was higher in later samples, with >95% ploidy consistency and 100% consistency of the sex chromosome. The primary explanation for these observations may be that the number of embryonic cells increases exponentially with in line with embryonic development and that the concentration of cfDNA surges dramatically at later stages of the development. Rubio et al. [28] transferred embryos into new culture drops onD4 and collected SCM on D5 to D7. The SCM consistency of D4-D6/7 was significantly higher than that of D4-D5 (84.0% vs. 63.0%), and the level of maternal contamination was also reduced. A multicentre clinical study which supports the above assumption. conducted by the same team that compared SCM and TE samples using a large sample size of 1,301 embryos, also achieved a remarkable 78.2% (866/1108) concordance rate using D4-D6/7 SCM samples [29]. While the consistency rate jumped to 92.0% if the culture were last until D6.

The above studies have shown that to maximize the collection of cfDNA content and ensure the detection rate and accuracy of cfDNA, there is an optimal collection time for SCM on the premise of ensuring the blastocyst rate and excellent embryo rate. For the fresh ET cycle, D4 to the pre-cryopreservation blastocyst stage (D5/D6/D7) could be the optimal collection time. Several studies of ni-PGTA have also been conducted on frozen-thaw embryos, where the culture media were collected at later stages and the assay performance was slightly better compared to that of the fresh embryos (as shown in **Figure 4**).

2.3.2 Fresh-frozen embryos yield better niPGT performance than fresh embryos

In cryopreservation of embryos, assisted blastocyst shrinkage is usually performed during vitrification of the embryo. Shrinkage of the blastocyst cavity before vitrification can prevent the formation of ice crystals and improve the survival rate of embryos after cryopreservation [44]. This process leads to lysis of cell membrane, which increases the likelihood of that embryonic cells releasing cytoplasmic materials, including genomic DNA, into the SCM, thereby increasing

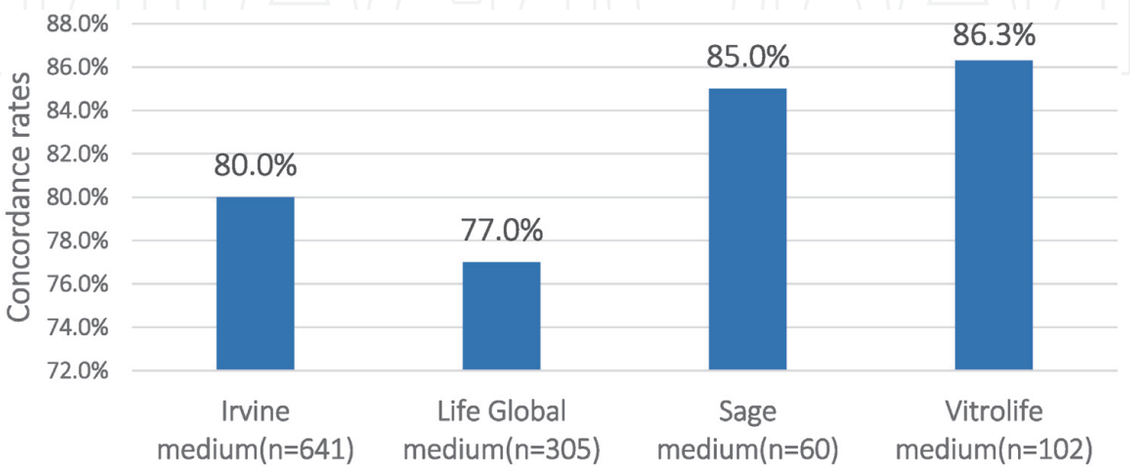

Figure 3.
CNV concordance rates of SCM versus TE biopsies in using different brands of culture media. No statistical differences were observed in four major brands of media. Data from Rubio et al. [42] (Ref). Data from Rubio et al. Embryonic cell-free DNA released to the spent blastocyst media. Am J Obstet Gynecol 2020.

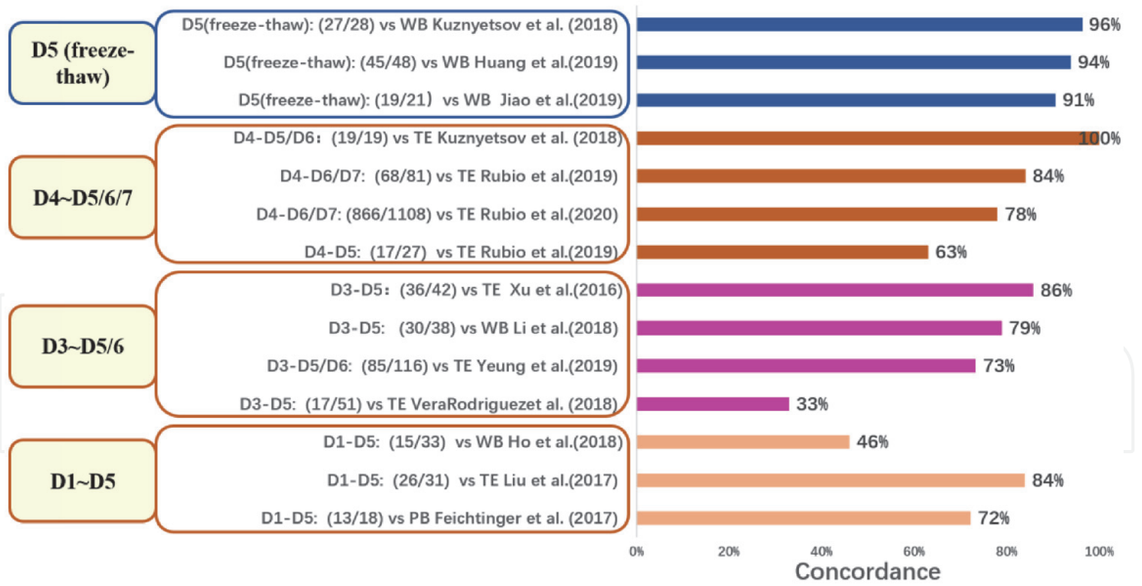

Figure 4.
Comparing SCM collection strategies and its impact on CNV concordance rate.

the concentration of DNA templates available for the downstream whole genome amplification (WGA) of niPGT-A. Furthermore, the vitrification process may lead to a higher rate of cell apoptosis, which also potentially increases the amount of cfDNA in the media after thawing and recovery. Kuznyetsov et al. [23] compared the amplification outcomes of a mixture of SCM and blastocyst fluid collected before vitrification (19) and after vitrification (30). The average amount of DNA obtained from fresh embryo culture amplification (6.3 ng/μL ~36.0 ng/μL) was lower than that obtained from vitrification-revival blastocysts (10.5 ng/μL ~44.0 ng/μL). After thawing 52 donated frozen blastocysts with TE biopsy results, Huang et al. [24] found that 48 of them (92.3%) were successfully amplified, and the data could be used for the analysis, with a consistency of 93.8% (45/48) to the whole embryo results. Jiao et al. [25] assessed 41 frozen blastocysts donated by 22 couples with known chromosomal rearrangements and 21 frozen blastocysts donated by 8 couples with normal karyotypes for PGT-SR and PGT-A analysis, respectively. All BCM (a mixture of SCM and BF) samples (62/62, 100%) were amplified successfully, and the clinical consistency between the niPGT-A results and the whole embryo was 90.48% (19/21). For PGT-SR results, the clinical consistency of BCM in chromosomal rearrangement and its corresponding whole embryo sample was 100% (41/41). Li et al. [26], using similar sampling methods, assessed 41 embryos and 97.6% were successfully amplified, and the clinical consistency between the niPGT-A results and the whole embryo was 87.2%. In summary, the SCM of cryopreserved-and-thawed embryos may contain more cfDNA than fresh embryos, which would minimize amplification failures and improve the reliability of the test results. This finding may be beneficial for patients who have had miscarriages due to embryo chromosomal abnormalities in the previous ART cycle. Providing that patients have sufficient cryopreserved embryos, they may take advantage of niPGT-A to screen euploid embryos for transplantation prior to the next transplantation cycle with resuscitated media (as shown in **Figure 4**).

2.3.3 Methods of whole-genome amplification

Given the challenge of low-concentration cfDNA in SCM, a WGA method with high uniformity and fidelity is required to amplify a small amount of genetic

material to accurately detect the genetic status of embryos. Different WGA techniques discussed in the chapter include multiple displacement amplification (MDA), multiple annealing and looping-based amplification cycles (MALBA) and Sureplex/Picoplex (as shown in **Figure 5**).

MALBAC has a unique characteristic similar to linear amplification, which can reduce the sequence-dependent bias exacerbated by exponential amplification [34]. Several niPGT-A studies have used MALBAC to amplify cfDNA in SCM, and the success rates ranged from 90.9% to 100% [24, 31, 35, 38]. In 2019, Jiao et al. developed NICS-INST technology based on MALBAC, which incorporated WGA and library preparation in the same amplification step, achieving a 100% (41/41) detection rate in SCM [25].

Picoplex/Sureplex is currently the most widely used WGA methods for conventional PGT-A. One study used Picoplex for SCM amplification with a success rate of 97% [37]. In another study using the Sureplex amplification method, the amplification rate of 22 SCM samples reached 81.8%, and all the amplified samples produced PGT-A results by aCGH [36]. A study using Sureplex WGA in combination with blastocyst fluid (BF) and SCM found amplification rates of 100% in 28 samples, all of which produced PGT-A results [23]. In one SCM study with a large sample size of 168, 89.3% of the samples were successfully amplified by Sureplex, and 77.3% of the sequenced samples yielded PGT-A results [34]. Lledo et al. compared Veriseq (Illumina®) and NICS (Yikon®), which both achieved 92.4% amplification success rates. The consistency of the culture medium collected on D6 and TE biopsy was 92.0% and 86.5%, respectively [30].

MDA is an isothermal amplification reaction using Phi29 DNA polymerase [45]. Amplification bias due to nonlinear amplification remains a significant fault in the technique. Studies using MDA on SCM have shown an amplification success rate of 97%; however, only 2% of the amplified samples produced reliable PGT-A results [39]. Since the cfDNA of SCM is dominated by short fragments with a length of 160–220 bp [46], this would especially affect the MDA-based method, which requires longer DNA fragments to achieve optimal amplification.

The Phi29DNA polymerase used in MDA technology has high fidelity but is restricted by the starting amount of the DNA template. When the starting amount is very low, the coverage rate and accuracy of the amplified products can decrease

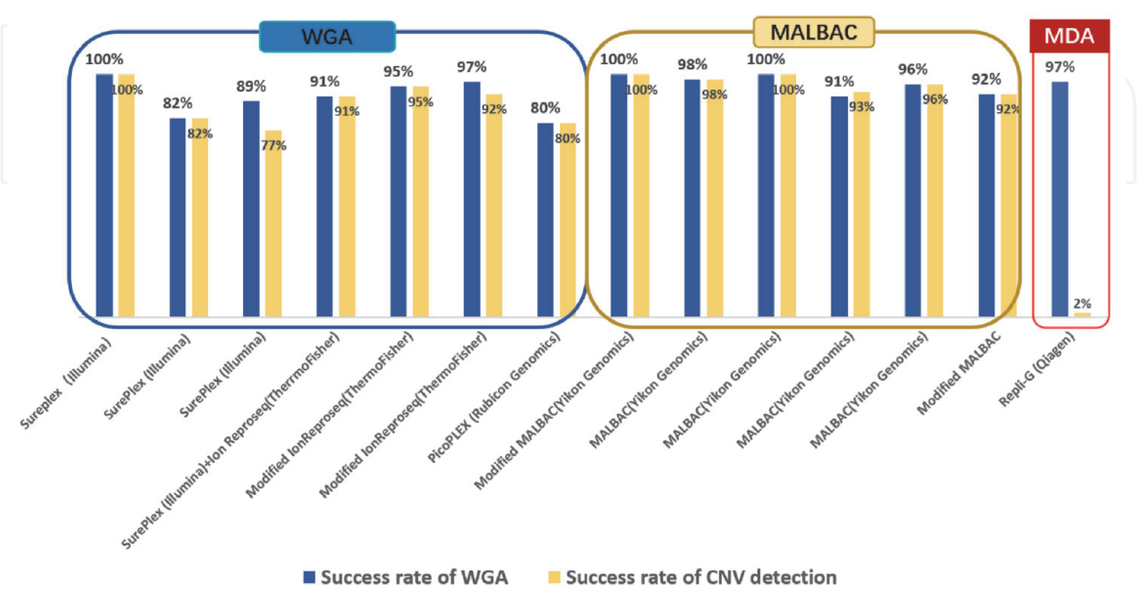

Figure 5.
Comparing SCM amplification methods and the concordance of CNV in different study MALBAC and SurePlex, PicoPLEX and IonReproseq are the main methods, MDA is not applicable.

dramatically. Malbac and Sureplex have the advantages of high genome coverage, low ADO rate, and a low number of starting DNA templates required. However, DNA polymerase has lower fidelity than Phi29 polymerase, which leads to an increased false positive rate compared to MDA.

2.3.4 Standard of the mosaic threshold of embryo

Embryonic mosaicism refers to the existence of two or more cell lines with different genotypes in the same embryo. Some studies suggest that abnormal cells in a mosaic embryo are gradually diminished, i.e., a certain degree of self-repair occurs during embryonic development [47–49]. Mosaic embryos, especially those with a low proportion, still have a decent success rate of pregnancy after being implanted [50, 51]. Therefore, there is a trend of accepting low-frequency mosaic embryos in clinical practice.

Chromosome ploidy of mosaic embryos can be quantified by the proportion of mosaics (M), and samples with results above a certain threshold of M are identified as aneuploidy embryos. Therefore, the proportion of false positives and false negatives in embryo chromosome detection relies on the threshold setting of M. We observed that different studies had different criteria for reporting mosaicism, ranging from 30% to 60%. Yeung et al. [34] set the minimum threshold as 30% and recommend against the implantation of mosaic embryos >30%. Bolton et al. [47] found that the elimination of aneuploidy cells in the ICM through apoptosis may result in an increase in aneuploidy fragments in the SCM. Thus, increasing the chimaerism threshold of SCM samples may reduce the false positive rate and improve the consistency rate. In the study of Jiao et al., samples with a mosaic ratio of less than 40% were defined as euploid, while samples with ≥40% were defined as aneuploid [25]. Rubio et al.'s study directly defined samples with a chimaerism ratio of <50% as euploidy and ≥ 50% as aneuploidy, and no mosaic embryos were reported [28]. Huang et al. observed that using 60% as the threshold for distinguishing aneuploidy from euploid embryos achieved a false negative rate of zero, significantly improving the results of non-invasive PGT-A [24]. This study suggests that appropriately increasing the aneuploidy threshold can minimize the mosaic false positives caused by NGS results to a certain extent; however, these adjustments would require validation on a much larger scale.

2.3.5 Whole embryo samples are more suitable as the gold standard for niPGT evaluation

Individual research groups may use different gold standards when evaluating the accuracy of SCM or BF detection results. At present, TE biopsy is the standard clinical practice for PGT-A; thus, TE biopsy is often used as the gold standard. However, due to the presence of chimaeric embryos, studies have questioned whether a couple of TE biopsy cells is able to accurately represent the ploidy of the inner cell masses. There was also a study performed to compare the ploidy results from cfDNA and polar body biopsy [36]. Although the polar body is naturally released from oocytes, which are minimally invasive, it only examines aneuploidy of maternal meiosis origin and is limited by its inability to directly evaluate the genetic status of the entire embryo [52].

Consequently, the D5 whole embryo for comparison with cfDNA as the gold standard may be the most appropriate when considering the accuracy of the given selected comparison criteria. Several studies have reported the use of the whole embryo as the gold standard. SCM collected from thawed blastocysts was assessed, which achieved overall consistency with the WB samples, ranging from 90.48% to 96.4% in all these studies [23–25]. Nevertheless, it can be challenging to obtain

donated WB, even though it may be the best representation of the genetic status of the entire embryonic genome.

2.3.6 The determination for concordance

The concordance between the SCM and the selected gold standard, e.g., conventional TE biopsy or whole embryo, can be analysed from several aspects, as shown in **Table 2**.

First, the statistics of the consistency rate can be grouped into the following three situations: (1) the negative consistency rate (euploidy vs. euploidy), that is, both SCM and gold standard are negative results reported as euploidy, which is also the most critical indicator in clinical practice. Generally, clinicians choose euploid embryos for transplantation, and the accuracy of this indicator often directly impacts the clinical outcome. (2) The positive consistency rate (aneuploidy vs. aneuploidy), that is, both SCM and gold standard are positive results reported as aneuploidy. Usually, clinicians would not choose such embryos for transplantation. Hence, the accuracy will directly affect the availability of embryos for transplantation and the cycle cancellation rate. (3) The overall consistency, which is a comprehensive evaluation including both euploidy and aneuploidy.

Second, the researchers likewise have different ways to calculate consistency. (1) Clinical consistency refers to the test results for the impact on the clinical decision, as some researchers suppose that only euploid embryos would be considered for implantation, which means clinical consistency is the foundation, which agrees with the transplant decision (as shown in **Figure 6**). (2) Full consistency: it can only be considered consistent when the test result is absolutely in line with the karyotype, especially for patients with known chromosomal abnormalities (as shown in **Figure 6**). (3) Partial consistency: Some researchers consider SCM and the gold standard to be two different types of samples. Thus, they should be considered as consistent if there is partial consistency with the karyotypes.

Third, the criteria for the reporting of aneuploidy are not uniform. Researchers usually consider whether to report fragmentation abnormalities or whole chromosome abnormalities and whether to report mosaic results and their reporting criteria.

Through the analysis of a series of related studies (see **Table 2**), we observed that although different investigators used different methods to calculate consistency, the consistency results were superior to other methods when only clinical consistency rates were considered.

In the studies of Huang et al. [24], Jiao et al. [25], Li et al. [35], Liu et al. [38], and Yeung et al. [34], when the karyotype was completely consistent or partially consistent, the results' coincidence rates to the gold standard were 83.3%, 76.2%, 50%, 64.5% and 62.1%, respectively. However, the consistency rates increased to 93.8%, 90.5%, 78.9%, 83.9% and 73.3%, respectively, when only clinical consistency was considered.

In addition, in the analysis of Kuznyetsov et al., if only chromosomal aneuploidy was considered, the clinical consistency rate was often better than the results of fragment abnormalities and consistent karyotypes. The clinical concordance of frozen embryos and fresh embryos was 96.4% and 100% without considering chimaerism and fragment abnormalities, respectively, but decreased to 78.5% and 73.7% when considering chimaerism and fragment abnormalities, respectively. In addition, only euploidy and aneuploidy were distinguished according to the chromosomal test results given, and the positive and negative consistency rates in each study were calculated.

Study	Concordance rate			Definition of concordance			Definition of aneuploidy			
	Concordance	PPV	NPV	Clinical concordance	Karyotype complete concordance	Karyotype partial concordance	Chromosomal level(mosaic)	Chromosomal level (No mosaic)	Chromosomal segment level (mosaic)	Chromosomal segment level (No mosaic)
Huang et al. [24]	93.8%(45/48) vs WB	91.7% (33/36)	100% (12/12)	√					√	
	83.3%(40/48) vs WB	91.7% (33/36)	100% (12/12)		√				√	
Jiao et al. [25]	90.5%(19/21) vs WB	75%(6/8)	100% (13/13)	√					√	
	76.2%(16/21)	75%(6/8)	100% (13/13)		√					
Xu et al. [31]	85.7%(36/42) vs TE	78.9% (15/19)	91.3% (21/23)	√					√	
Li et al. [35]	78.9%(30/38) vs WB	73.9% (17/23)	86.7% (13/15)	√					√	
	50%(19/38) vs WB	73.9% (17/23)	86.7% (13/15)		√				√	
Liu et al. [38]	83.9%(26/31) vs WB	80%(8/10)	90% (18/20)	√					√	
	64.5%(20/31) vs WB	80%(8/10)	90% (18/20)		√				√	
Rubio et al. [28]	78.7%(85/108) vs TE	77.6% (52/67)	91.7% (33/36)	√						√
Yeung et al. [34]	73.3%(85/116)	82.6% (71/86)	46.7% (14/30)	√					√	
	62.1%(72/116) vs TE	82.6% (71/86)	46.7% (14/30)			√			√	
Vera-Rodriguez et al. [41]	33.3% (17/51) vs TE	90.9% (20/22)	20.7% (6/29)	√					√	

Study	Concordance rate			Definition of concordance			Definition of aneuploidy			
	Concordance	PPV	NPV	Clinical concordance	Karyotype complete concordance	Karyotype partial concordance	Chromosomal level(mosaic)	Chromosomal level (No mosaic)	Chromosomal segment level (mosaic)	Chromosomal segment level (No mosaic)
Kuznyetsov et al. [23]	96.4% (27/28)vs WB	100% (21/21)	85.7% (6/7)	√			√			
	92.8%(26/28) vs WB	100% (21/21))	85.7% (6/7)		√		√			
	78.5%(22/28) vs WB	79.2% (19/24)	75%(3/4)		√				√	
	100% (19/19) vs TE	100% (8/8)	100% (11/11)	√			√			
	100%(19/19) vs TE	100% (8/8)	100% (11/11)		√		√			
	73.7%(14/19) vs TE	72.7% (8/11)	75%(6/8)		√				√	

Table 2.
The different definitions of concordance and the aneuploidy affect the concordance rate.

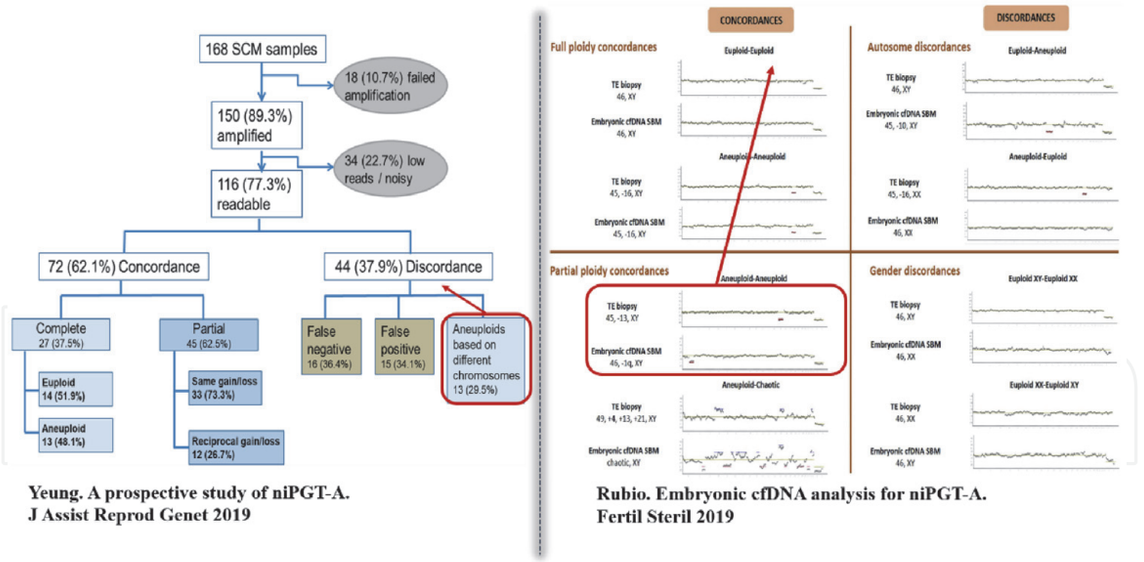

Figure 6.
Definition of concordance between SCM and the gold standard are at different levels in studies. Yeung et al. defines concordance at chromosome level, while Rubio et al. defines at the level of clinical concordance.

In general, the negative consistency rate is better than the positive consistency rate in SCM, which may indicate high false positives in the niPGT-A test. On the other hand, it also shows that the negative result of this approach is more reliable. In summary, the definition of consistency has a direct impact on the final results of the assessment method. Considering the speciality of cfDNA presented in SCM, it seems insufficient to define consistency purely based on the perfect match of the CNV patterns. Chen et al. [27] proposed the embryos can be categorized or scored by the obtained niPGT-A results into three groups: (A) chromosomal normal, (B) chromosomal abnormal, and (C) multiple abnormal chromosomes or uncertain. The results showed group A predicts a normal embryo with 90.0% probability, while groups B and C predict 27.8% and 72.2% normal probability, respectively. Thus, it may be necessary to fully consider the conditions, such as mosaicism, ploidy status and test resolution, when establishing the analytical model.

It seems more appropriate to use whole blastocysts as the gold standard for evaluation of the accuracy of niPGTA. Apparently, the establishment of the model would require large embryos of SCM and WB paired samples, with standardized sample collection and data analysis procedures. Furthermore, the model can evaluate SCM according to the euploidy probability of embryos to obtain the priority or exclusion order of embryos for implantation to avoid the waste of embryos due to false-positive decisions led by conventional PGT-A.

2.4 Clinical application of non-invasive PGT-A

At present, several studies have applied non-invasive PGT-A to diverse patient groups to preliminarily evaluate the clinical manifestations of the technique. Xu et al. [31] used non-invasive PGT-A for the first time in balanced translocation patients and obtained five live births from seven couples. Rubio et al. [28] compared the clinical outcomes of two groups of patients, one with both TE biopsy and SCM results of euploidy and the other with TE biopsy negative and SCM results of aneuploidy. The transplant success rate of patients with both euploidy TE and SCM results was twofold (52.9% vs. 16.7%) higher than that of the latter group. Zero miscarriages were observed (0/9) when both the TE and SCM results indicated that

the embryos were euploid. Moreover, Fang et al. [53] reported the results of a single-centre clinical trial in 2019 applying non-invasive PGT-A on patient groups with either repeated implant failures (≥3) or repeated miscarriages (≥3). The trial included 45 couples with 50 ART cycles, resulting in a biochemical pregnancy rate of 72% (36/50), a clinical pregnancy rate of 58% (29/50), and a spontaneous miscarriage rate of ~10% (3/29), with a total of 27 babies successfully delivered. While the clinical trial scale of the above studies was small, the cfDNA-based niPGT-A proved in principle that it could reduce miscarriage and improve the ongoing pregnancy rate. Conversely, large-scale randomized controlled clinical trials are needed to conclude whether non-invasive PGT-A can be an effective way to evaluate embryo implantation potential.

3. Studies of cfDNA in blastocyst fluid (BF)

3.1 Presence of cfDNA in in blastocyst fluid (BF)

The presence of cfDNA in blastocyst fluid (BF) was revealed as early as 2013. For the first time, Palini et al. [17] reported that genomic DNA has identified in 90% (26/29) BF by qPCR, with an average amount of gDNA at 9.9 pg. TBC1D3 on chr17 and TSPY1Y on the Y chromosome were successfully amplified, which confirmed that the sex of embryos could be determined by qPCR using BF. In addition, genome-wide amplification (WGA) was demonstrated for blastocyst fluid amplification, and aneuploidy detection of WGA products was performed using microarray comparative genomic hybridization (aCGH). The aCGH results revealed the presence of the Y chromosome in two BF samples, which was consistent with previous data obtained by PCR analysis. It also showed multiple chromosomal abnormalities that agreed with the karyotype, suggesting that the WGA product DNA was indeed of embryonic origin. Subsequently, Zhang et al. [46] performed next-generation sequencing on WGA-amplified BF DNAs and compared the data with paired blastomere biopsy samples. The results showed decent concordance in genomic coverage and pattern regions between the two DNA sources. Further analysis of the gene annotation results suggested that cfDNA in the BF contained the sequences of the majority of genes, indicating that cfDNA in the BF could be used for solving monogenic diseases.

3.2 Sampling method of blastocyst fluid

BF can be isolated from embryos for downstream analysis using blastocyst puncture, which is a microscopy-assisted puncture made with an ICSI injection pipette on the trophoblast side, opposite of the inner cell mass. The fluid is then taken until the blastocyst completely collapses around the pipette [54]. This method can only isolate minute amounts (~0.01 μL) of BF for analysis [55, 56].

3.3 Study of PGT-A using blastocyst fluid

Recently, BF has been studied as a source for minimally invasive PGT-A. In 2014, Gianaroli et al. [57] isolated BF from 51 blastocysts, and the average concentration of WGA-amplified DNA detected in 39 BF samples (76.5%) was 900.38 ng/μL (ranging between 876.3–939.5 ng/μL). The results of BF detected in 38 cases (38/39, 97.4%) were consistent with the ploidy of TE biopsy results, and 9 cases (9/9, 100%) were consistent with the ploidy of the blastomere biopsy, and the coincidence rate of PB ploidy was 93.3% (28/30). Magli et al. [55] of the same group

improved the sampling and amplification of BF samples to a success rate of 82% (95/116). aCGH was performed on 87 of these samples, of which 82 samples (94%) were used for subsequent analysis. The ploidy consistency of BF and TE samples was 97.1% (67/69), 94.4% (34/36) with blastomere biopsy and 94.1% (32/34) with polar body biopsy. In 2019, Magli et al. [12] used the same approach to amplify blastocyst fluid extracted from 256 blastocysts with a 71% (182/256) WGA amplification rate. Compared with the results of the corresponding TE cells, the overall ploidy consistency was 93.6% (161/172). The above results suggested that the cfDNA of the BF was highly predictive of embryo ploidy; however, the results from other research groups have been less satisfactory.

Tobler et al. [58] obtained a WGA amplification success rate of 63% (60/96) after thawing and culturing donated embryos to extract BF. The concordance between the BF karyotype and the whole embryo was 48.3% (29/60) by aCGH analysis, suggesting that BF may not be suitable for the PGT test. In 2018, Tsuiko performed chromosome analysis using NGS on 16 donated blastocysts, and the success rate of BF amplification was 87.5% (14/16). However, only 10 samples (10/16, 62.5%) passed sequencing and quality control for the subsequent analysis. The results showed that only 40% (4/10) of the BF samples completely agreed with the TE or ICM results. In addition, significantly more mosaicisms were found in BF samples than in TE and ICM samples. These results together suggest that although BF-DNA can be successfully amplified for NGS, one would not recommend using BF as a single DNA source for preimplantation genetic screening due to its low concordance with ICM and TE results. Capalbo et al. [59] (2018) performed PGT-A on 23 BF samples and compared the results with TE. Only 8 cases (34.8%) were successfully amplified, and only 3 cases (37.5%) were consistent with the ploidy of TE detection results.

In summary, studies assessing the reliability of BF DNA as a template for PGT-A have yielded conflicting conclusions, with 37.5%–97.4% concordance with TE samples (**Table 3**). Differences in consistency levels between studies may be related to differences in embryo handling. Tsuiko and Tobler used frozen embryos donated by patients after treatment, and BF was absorbed after blastocyst resuscitation and expansion; Tobler's study [58] specifically suggests that they are not suitable for clinical use. In contrast, the BF samples of Gianaroli, Magli and colleagues were obtained from fresh culture cycles and underwent many procedures (PB biopsies, assisted incubation and blastomere biopsies) that were not performed or relatively few procedures were performed in other studies. Thus, the observed increased rates of amplification and concordance may be due to the superior quality of BF obtained from freshly cultured embryos over frozen embryos or to an increase in the amount of DNA in the blastocyst cavity resulting from unintentional cell lysis or death during the procedure. It is also important to point out that the sample size in the Capalbo and Tsuiko studies was much smaller than that of Gianaroli, Magli and colleagues, so different results might have been obtained if a larger sample size had been studied.

According to the results of available studies collectively, BF DNA presented a high percentage of DNA amplification failure. The percentage of blastocyst fluid samples that successfully underwent whole genome amplification and produced detectable levels of DNA ranged from 34.8% to 82% [46, 58–60]. The difficulty in the successful amplification of BF-DNA lies in the small amount of fluid obtained from the cystic cavity, and the BF volume reported in various studies ranged from 0.3 nl to 1 μl. The volume difference would significantly impact the concentration of BF-DNA and may impose a negative effect on the efficiency of subsequent amplification. Another major reason for failure in BF-DNA amplification would be degradation. The success rate of whole genome amplification was generally much higher (≥98%) [58, 61] in TE biopsy, which makes BF DNA less suitable as an alternative source for clinical application [19].

Study	Fresh/frozen embryos	Method of BF collection	Day of BF isolation	Volume used of WGA	WGA method	WGA products detection%(n)	DNA analysis	Concordance, %(n)
PRO								
Gianaroli et al. [57]	Fresh	Blastocentesis	D5	1 μL	Sureplex	76.5%(39/51)	aCGH	100% vs BM(9/9) 97.4% vs TE (38/39) 93.3% vs PB(28/30)
Magli et al. [55]	Fresh	Blastocentesis	D5	0.01 μL	SurePlex	82%(95/116)	aCGH	94.4% vs BM(34/36) 97.1% vs TE (67/69) 94.1% vs PB(32/34)
Magli et al. [60]	Fresh	Blastocentesis	D5	The aspirated fluid was transferred into a 1-μL droplet of PBS	SurePlex	71%(182/256)	aCGH	93.6% vs TE (161/172)
CON								
Tsuiko et al. [56]	Cryopreserved blastocysts	Blastocentesis	D5	0.01 μL	Picoplex	62.5% (10/16)	NGS	40% vs ICM 或TE(4/10)
Tobler et al. [58]	Cryopreserved blastocysts	Blastocentesis	D5	1 μL	SurePlex	63%(60/96)	aCGH	48.3% vs EB(29/60)
Capalbo et al. [59]	Fresh	Blastocentesis	D5	The aspirated fluid was transferred into a 5-μL droplet of medium	SurePlex	34.8%(8/23)	NGS	37.5% vs TE(3/8)

Table 3.
Summary of BF cfDNA study.

In addition, blastocyst puncture is still a minimally invasive procedure, as an ICSI needle is inserted into the blastocyst cavity to extract the fluid. However, with the presence of amplifiable cfDNA in the BF, the low consistency of the test results to TE or other gold standards suggests that technical variability may still exist. The process may accidentally acquire loose cells trapped in the lumen or shed cell material, resulting in inconsistent test outcomes. Thus, further optimization is required before using BF DNA for PGT.

3.4 Correlation between BF test results and clinical outcomes

The DNA concentration in the BF was very low, and the total amount varied greatly between samples, implying that the BF DNA amount could be related to the developmental status of embryos. In 2019, Magli et al. [60] showed a significantly higher WGA amplification success rate of BF in aneuploid blastocysts (n = 150, 81%) versus euploid blastocysts (n = 32, 45%), suggesting differences in the quality or total amount of DNA in the BF of euploidy and aneuploidy embryos. Moreover, the clinical pregnancy rate was 77% in the group with failed BF amplification and 37% in the group with successful BF amplification when following the clinical outcome of 53 TE euploid blastocysts. The same trend was found in the rate of persistent pregnancies in failed and successful amplified BF samples, which were 68% and 31.5%, respectively. These clinical results suggest that the success of BF amplification may have predictive value for the viability of the corresponding blastocyst; nevertheless, further validation is required to draw conclude. Given that the total amount of DNA in the BF is relatively low, combining blastocyst fluid and culture medium may increase the amount of cfDNA, therefore improving the amplification success rate and consistency of detection.

4. Problems in the application of cfDNA to PGT-A

Before the large-scale use of SCM in PGT-A, further investigations are needed to trace the origin of cfDNA and confirm whether cfDNA represents the euploidy of embryos.

4.1 The origin of cfDNA

At present, there are several opinions about the origin of cfDNA. It is believed that during embryonic development, cfDNA may be released into the culture media through cell lysis, apoptosis, cell debris or other mechanisms [19]. In apoptotic cells, DNA is cleaved to form fragments. These fragment sizes are multiples of approximately 180-bp oligomers [62]. Zhang's [46] research shows that the fragments have two peaks. The first peak had a range of 160–220 bp, whereas the second peak was broader, ranging from 300 to 400 bp. Bolton et al. [63] found that apoptosis was frequently observed within the ICM and TE between euploid cells and aneuploid cells in a mouse model. The results also showed that in aneuploid embryos, the percentages of apoptotic cells in the ICM and TE were 41.4% and 3.3%, respectively. In euploid embryos, the percentage of apoptotic cells in euploid cells was 19.5% and 0.6%, respectively. If cfDNA mainly comes from apoptotic cells, Bolton's and other studies demonstrate that cfDNA mainly originates from the ICM.

Moreover, Victor et al. [51] tracked the development of embryos dynamically using immunofluorescence technology, especially cell proliferation and apoptosis in euploid embryos, aneuploid embryos and mosaic embryos. The TE and ICM in euploid cells showed lower level of activities in both not only in cell proliferation

but also in apoptosis. Nevertheless, in mosaic and aneuploid embryos, the levels of the two processes were both higher.

4.2 Maternal contamination

The discordant results in SCM and control embryos were mainly attributable to a high percentage of maternal DNA in the spent culture media. The Vera Rodriguez team [41] analysed the SCM and TE results from 56 samples. Among them, 17 embryos were detected as aneuploid or aneuploid males (XY) by TE, while all of them were detected as aneuploid females (XX) by SCM. When Feichtinger et al. compared the consistency between the SCM and polar body, the negative control (SCM of fertilization failure oocytes) was also effectively amplified. Oocytes are unlikely to abandon their DNA to the culture media. Therefore, this maternal contamination may come from cumulus cells or other exogenous DNA [36]. However, contamination may be minimized with the degradation of DNA in vitro by changing the solution in the sequential culture at D3 and delaying the sampling time. In sequential culture, if the granular cells before ICSI are not removed entirely, it is recommended to remove them again during the culture medium change on Day 3.

To reduce the interference of human judgement, we should develop a calculation method to eliminate maternal DNA for the purpose of internal quality control. To confirm whether there was maternal DNA contamination in the embryo medium, Hammond et al. carried out a short tandem repeat (STR) analysis on abandoned spent culture media (n = 10), media controls (n = 2) and the corresponding cumulus cells [64]. Maternal DNA from cumulus cells successfully amplified all gene loci, but the amount of DNA in the SCM was too small to identify DNA. STR analysis is a long-range amplification, and highly fragmented SCM DNA seems unsuitable for this detection. Vera Rodriguez et al. conducted SNP sequencing of three groups of samples (TE/follicular fluid DNA/embryonic SCM DNA) from 35 embryos and successfully quantified the proportion of maternal DNA contamination in SCM [41]. This suggests that SNP detection can be used to evaluate maternal contamination in SCM.

In addition, considering that the fragment size of cfDNA may be different from that of maternal contamination DNA, or according to the cleavage characteristics of cfDNA at the restriction site, it can be considered to identify the target fragment size and restriction site characteristics, and only analyse the DNA that conforms to the embryonic DNA. The hypothesis is that there is a DNA amplification and database construction method that can maintain the characteristics of DNA fragments or restriction sites, and the original cfDNA template can be amplified with high fidelity.

4.3 Background cfDNA contamination of SCM

Some studies have suggested that another source of exogenous DNA contamination is the low level of background DNA in SCM. In 2017, Hammond et al. [64] detected low baseline levels of DNA in the base media and protein supplement components of three commercial culture media. They also detected a copy of nuclear DNA in the culture media with no previous contact with embryos. Vera Rodriguez et al. [41] detected DNA quantification using qPCR in 53 spent culture media and 17 control samples (culture media with no previous contact with embryos). Then, PGT-A analysis was performed in 56 spent culture media and 11 control samples. The SCM was amplified successfully, and 11 controls generated an amplification-failure pattern, causing downstream analysis failure. In addition, Li et al. [35] found that the background DNA of the control culture did not interfere

with the detection results, and all samples from the same embryo had the same sex chromosome diagnosis. The results testified that although there was a low baseline of exogenous DNA in the control SCM, the effect could be ignored due to the small amount of exogenous DNA. In addition, some commercial media use human serum albumin (HSA) to improve embryo development. Because HSA can adsorb DNA, the amount that is added should be controlled within a reasonable range. We suggest that the blank culture medium should be set as the parallel culture at the same time as recovering blastocyst culture media for quality control of SCM samples.

5. Prospects

Using SCM cfDNA for PGT-A has become a hot topic in the assisted reproductive field. The current research focuses on the aneuploidy consistency between cfDNA and embryos. However, the consistency comparison needs to consider many influencing factors, such as the definition of consistency, sampling methods, analysis methods, etc. We need to reach a certain consensus and standard before making an objective evaluation. Through the summary and introduction of this review, we found that a suitable sampling time, WB as the gold standard, suitable amplification methods and NGS platforms may provide the basis for the standardization of non-invasive PGT-A.

In the future, we should develop a standardized and automatic embryo culture and sample collection system based on the operating habits and culture process of the embryo laboratory. Meanwhile, we need to combine the sample downstream detection technology with corresponding software analysis to select the best embryos, which are those with the lowest probability of chromosomal abnormalities, for transplantation to improve the success rate of the whole IVF-ET cycle. It is believed that with the development of technology, the realization of automatic collection and detection platforms, the accumulation of detectable sample sizes, and the average cost of PGT-A detection on each embryo will be greatly reduced, and it is encouraging that SCM sample collection and detection will be developed into the routine process of embryo laboratories.

With the accumulation of data, SCM can be used to develop detection content for different populations. For the low-risk population with chromosomal abnormalities, we can give the risk value of chromosomal abnormalities on chromosome 16, which easily causes abortion, and on chromosomes 13, 15, 18, 21 and X, which easily cause birth defects. Referring to the NIPT method, the influence, caused by a proportion of false positive cfDNA, will be reduced. For high-risk populations with chromosomal abnormalities, such as elderly individuals, the consistency between SCM test results and embryos is high, and the interference of false-positives is relatively low. For this group of people, it is recommended to conduct whole chromosome screening, determine the risk value of chromosomal abnormalities of each embryo and determine the order of embryo transfer according to the risk value. For people with clear indications of PGT-A, we suggest that SCM samples be stored while biopsy samples are collected, especially for the laboratory or biopsy operator who has just performed biopsy. If there is no result or the result of biopsy cannot be used, SCM test results could provide remedial measures for the failure of detection due to sample loss or operation. In addition, for IVF cycles with poor quality or a small number of embryos, we should minimize the damage of biopsy operations to embryos and consider the detection of chromosome aneuploidy by SCM under the premise of full knowledge.

Exogenous contamination of embryos has been a more concerning issue at present. To improve the accuracy of detection, DNA fragments from embryos should be identified by differentiated DNA fragment length or linkage analysis in a

bioinformatics platform. In addition, due to the different fertilization methods of IVF embryos, researchers have been worried that sperm will interfere with SCM. If we find a way to eliminate maternal or paternal contamination, it will be supplied to IVF embryos. We can use this technology to eliminate the problem of chromosome aneuploidy through non-invasive PGT-A to achieve ideal clinical outcomes for a wider population.

At the same time, non-invasive PGT detection can try not only for aneuploidy detection but expand to the comprehensive evaluation on the based on morphology and take into account the DNA concentration, chimaeric ratio, resolution, consistency with the gold standard and other factors of embryonic SCM. With the accumulation of clinical outcomes, it can also be combined with the clinical data of patients as an index to predict the clinical outcomes of embryos.

A noninvasive artificial intelligence embryo evaluation model could be established, which not only provides suggestions for clinicians on the order of embryo implantation but also provides patients with the most suitable and economical detection scheme, ultimately saving time for pregnancy and improving the overall success rate of IVF-ET.

6. Conclusions

As the findings of non-invasive PGT-A (NIPGT-A) research have been discussed, and researchers have noticed that the success rate and accuracy of the test are closely related to the types of culture medium, culturing and methods, sampling method, detection platform s and gold-standard reference used of detection. This chapter systematically expounds on how researchers use foetal free DNA for non-invasive PGT-A detection and thoroughly analyses the factors affecting its accuracy, possible problems and future application prospects. We hope that it serves as a good reference for non-invasive PGT-A being widely used in standardized operations before clinical application.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Acronyms and abbreviations

PGT-A	Preimplantation Genetic Testing For Aneuploidy
cfDNA	Cell-free DNA
niPGT-A	non-invasive PGT-A
IVF-ET	In vitro fertilization and embryo transfer
eSET	elective single embryo transfer
BCM	blastocoel fluid and spent culture medium
SCM	spent culture media

BF	blastocoel fluid
gDNA	genomic DNA
mtDNA	mitochondrial DNA
TE	trophectoderm
NICS-INST	Non-invasive chromosome screening: an improved MALBAC whole genome amplification (WGA) strategy
PGT-SR	Preimplantation Genetic Testing for Structural Rearrangement
WGA	whole genome amplification
MDA	multiple displacement amplification
MALBAC	multiple annealing and looping-based amplification cycles
NICS	Non-Invasive Chromosome Screening
ADO	allele dropout
NGS	next-generation sequencing
WB	whole blastocyst
CNV	copy number variations
aCGH	array-based comparative genomic hybridization
ICSI	Intracytoplasmic sperm injection
PB	Polar body
STR	short tandem repeat
SNP	single nucleotide polymorphism
HSA	Human serum albumin

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