

Is cell-free DNA in spent embryo culture medium an alternative to embryo biopsy for preimplantation genetic testing? A systematic review

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Title: Is cell-free DNA in spent embryo culture medium an alternative to embryo biopsy for preimplantation genetic testing? A systematic review.

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Conflict of interest

The authors have no conflict of interest concerning this manuscript.

ABSTRACT

Preimplantation genetic testing (PGT) is increasingly used worldwide. Currently, it entails the use of invasive techniques (i.e., polar body/blastomere/trophectoderm biopsy or blastocentesis) to obtain embryonic DNA, with major technical limitations and ethical issues. There is evidence to suggest that invasive PGT can lead to genetic misdiagnosis in the case of embryo mosaicism, and consequently to the selection of affected embryos for implantation or to the destruction of healthy embryos. Recently, spent culture medium (SCM) has been proposed as an alternative source of embryonic DNA. An increasing number of studies have reported the detection of cell-free DNA (cf-DNA) in SCM, and highlighted the diagnostic potential of non-invasive SCM-based PGT for assessing the genetic status of preimplantation human embryos obtained by *in vitro* fertilization (IVF). However, the reliability of this approach for clinical applications needs to be determined. In this review, we present the published evidence on non-invasive SCM-based PGT, and its current benefits and limitations compared with iPGT. Then, we discuss how to optimize and standardize procedures for non-invasive SCM-based PGT to prevent technical biases and to improve performance in future studies. Finally, we present the clinical perspectives of non-invasive PGT and highlight its future applications in reproductive medicine.

Introduction

Preimplantation genetic testing (PGT) is used to test human embryos obtained by *in vitro* fertilization (IVF) to detect monogenic/single-gene defects (PGT-M), chromosomal structural rearrangements (PGT-SR), or aneuploidy (PGT-A) with the aim of selecting unaffected embryos for implantation. This should increase the odds of having a healthy baby.

PGT, which was introduced in 1989 (Handyside et al. 1990; Handyside et al. 1989), has now become a routine clinical procedure and is increasingly used worldwide (De Rycke et al. 2015). Currently, PGT relies on invasive approaches to obtain DNA from embryos generated by IVF (Figure 1A). Embryo biopsy may be performed at the cleavage stage (i.e., removal of one to two blastomeres) or at the blastocyst stage (i.e., removal of 5-10 trophectodermal cells) (Ray et al. 1996; Harton et al. 2011; Kokkali et al. 2005). Both techniques present technical advantages and disadvantages. Blastomere biopsy involves the micromanipulation of a high number of cleaved embryos among whom many will not reach the blastocyst stage; however, it gives adequate time for testing and embryo selection for fresh transfer (and/or embryo vitrification). Conversely, trophectoderm biopsy relies on the micromanipulation of few blastocysts with high implantation potential. However, it usually requires the vitrification of all (healthy and affected) biopsied blastocysts while waiting for the genetic test results, and consequently the transfer of thawed embryo(s). In some countries where embryo biopsy is not allowed, the removal of the first and second polar body at the zygote stage could represent an alternative (Montag et al. 2013). However, its usefulness for PGT is still a matter of debate because: 1) it does not allow assessing the paternal genetic contribution (Montag et al. 2013; Cimadomo et al. 2016); 2) it is expensive and time-consuming because it requires the biopsy of a high number of oocytes among which many will not be fertilized or will not reach the blastocyst stage (Cimadomo et al. 2016); and 3) it is associated with an increased risk of embryo misdiagnosis due to the high incidence of post-

meiotic chromosome abnormalities (Capalbo, Bono, et al. 2013; McCoy 2017). Therefore, oocyte/zygote biopsy is infrequently used nowadays (Cimadomo et al. 2016).

Although embryo biopsy is currently the preferred method worldwide, it has also major limitations. First, embryo biopsy can only be performed at a specific time point during embryo growth. Indeed, day 3 (D3) cleaved embryos with fewer than six blastomeres are not suitable for biopsy because blastomere removal affects underdeveloped embryos (Milachich 2014; Harton et al. 2011). Likewise, the number of biopsied cells negatively affects the implantation potential of blastocysts with poor trophectoderm quality (Zhang, Luo, et al. 2016). Moreover, early blastocysts cannot be biopsied because embryoblast cells may be unintentionally aspirated due to the proximity between trophectoderm and inner cell mass (Milachich 2014; Harton et al. 2011). Consequently, some healthy but underdeveloped embryos will not undergo biopsy, although they display some reproductive potential. Second, embryo biopsy involves the removal of only few cells from the whole embryo, and this might lead to genetic misdiagnosis (i.e., false positive/negative) in the case of embryo mosaicism (Vera-Rodriguez and Rubio 2017; McCoy 2017; Taylor et al. 2014; Sachdev et al. 2017). Third, invasive procedures are detrimental to the embryo reproductive potential. Indeed, biopsy at the cleavage stage leads to suboptimal embryo development (Kirkegaard, Hindkjaer, and Ingerslev 2012; Munne et al. 2007; Scott et al. 2013), with potential long-term detrimental effects in the neonates and children (Middelburg et al. 2010; Middelburg et al. 2011; Bay et al. 2016). Moreover, epigenetic modifications, neurodegenerative disorders, abnormal development of adrenal glands and ovary dysfunction have been reported in animal offspring obtained by IVF followed by embryo biopsy (Zhao et al. 2013; Yu et al. 2013; Yu et al. 2009; Wu et al. 2014; Zeng et al. 2013). Likewise, there are concerns about the safety of trophectoderm biopsy because it requires *in vitro* culture until the blastocyst stage (Braakhekke et al. 2015). It has been suggested that extended embryo culture beyond the

embryonic genome activation stage might increase the risk of monozygotic twins, perinatal mortality, congenital anomalies, preterm birth and large-for-gestational-age newborns (Martins et al. 2017; Hviid et al. 2018; Dar et al. 2014). Fourth, invasive procedures are expensive and time-consuming. These challenging techniques require advanced technical skills, constant training, and adequate material (e.g., laser equipment) (Milachich 2014). For all these reasons, alternative PGT methods are needed.

Recently, the detection of cell-free nucleic acids (cf-DNA) in biological fluids has opened new perspectives for the development of non-invasive tests in reproductive medicine (Lo et al. 1997; Traver et al. 2014; Assou et al. 2014; Stigliani et al. 2013). Indeed, cf-DNA has been detected in blastocoel fluid (BF) (Palini et al. 2013) and in spent culture medium (SCM) (Stigliani et al. 2013) of developing embryos generated by IVF (Figures 1B and 1C). However, BF sampling for cf-DNA has some potential drawbacks. Indeed, this approach must be considered as minimally invasive because the continuity of the trophectoderm layer is broken by the introduction of the injection needle for BF aspiration (i.e., blastocentesis) or by the laser pulse to release the BF into the culture medium. Moreover, it should be performed in expanded blastocysts to collect or release an adequate volume of BF (Palini et al. 2013). Consequently, early blastocysts (i.e. B1 and B2 (Alpha Scientists in Reproductive and Embryology 2011)) are unsuitable for this approach, although they display a fair implantation potential. In addition, the technical skills required for BF collection with an injection needle could lead to suboptimal procedures that affect the embryo reproductive competence. Finally, it still requires long-term biosafety testing.

Consequently, cf-DNA from SCM appears to be the best option for non-invasive PGT (Figure 1C). Indeed, the detection of nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) in SCM of human embryos during IVF procedures opens the possibility of using this non-invasive technique to develop a new PGT approach (Assou et al. 2014; Gianaroli et

al. 2014; Stigliani et al. 2013). Many studies have already reported the detection of cf-DNA in SCM and evaluated the potential of this approach to determine the genetic status of developing embryos. Therefore, a systematic review is now necessary to determine the relevance of SCM as source of cf-DNA for human embryo genetic testing. The first objective of this review was to present the evidence published to date on cf-DNA presence in SCM, using cf-DNA detection rate and quantification as endpoints. The second objective was to evaluate the reliability of non-invasive PGT based on SCM (SCM-PGT throughout the text) for determining the genetic status of embryos generated by IVF, using as endpoint the concordance rate of the results obtained by SCM-PGT and invasive PGT (iPGT). Finally, the clinical benefits and limitations of non-invasive PGT will be discussed. Moreover, an optimal non-invasive SCM-PGT procedure will be proposed to limit the technical biases and to improve the standardization of future clinical studies. Finally, the clinical perspectives and future applications of non-invasive PGT in reproductive medicine will be discussed.

Methods

The systematic review was performed following the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines (Liberati et al. 2009).

Registration

The protocol was registered in the International prospective register of systematic reviews (PROSPERO); the registration number is CRD118686.

Literature search

The following search terms were used to search the MEDLINE database from inception to December 2018: (preimplantation genetic diagnosis OR PGD OR preimplantation screening OR PGS OR preimplantation genetic testing OR PGT OR embryo screening OR cell-free DNA OR embryonic DNA) AND (culture media OR culture medium OR embryo media OR embryo medium). Additional studies were extracted from the references of the full-text articles.

Study selection

Articles were restricted to English language only. Two reviewers (SB and CC) independently reviewed the retrieved articles to exclude citations deemed irrelevant by both observers. The study eligibility was screened first by using the article titles, and then by reading the abstracts. Full texts of potentially relevant articles were retrieved and evaluated for inclusion. Any disagreement was resolved by discussion. The final decision was taken by the senior investigator (SH).

Data extraction

The following details were extracted to characterize the included studies: study authors, publication year, type of DNA, sample size, fertilization technique, nature of samples, amplification and DNA quantification method, genetic DNA analysis, detection/amplification rates, embryo DNA reference, general and full concordance rates. In addition, the features that

could potentially affect the quantity or quality of cf-DNA present in SCM, such as SCM collection timing, incubation time of the medium with the corresponding embryo, embryo stage and quality, any embryo micromanipulation before SCM collection, and embryo vitrification/thawing, were collected. Data extraction was independently performed by two reviewers (SB and CC). Any disagreement or uncertainty was resolved by discussion.

Quality assessment

Three reviewers (SB, CC and GM) assessed the methodological quality of each study using a modified Newcastle– Ottawa scale (mNOS) (Supplementary Table I and II).

Each study was rated according to six items categorized in three domains: selection, method and outcome (maximum scores of 2, 6 and 4, respectively). Scores were represented with stars for each quality item to provide a visual assessment. Studies were awarded up to twelve stars if they fulfilled all the quality items. No cut-off score was set for inclusion in this systematic review.

Results

Study selection

The study flow chart is presented in Figure 2. The MEDLINE database search for studies on cf-DNA in SCM of embryos generated by IVF yielded 8,309 publications. In total, 173 abstracts were reviewed, and 33 full-text articles were assessed for eligibility. After excluding the articles that did not address the research question, 15 articles were included in this systematic review.

Study characteristics

The characteristics of the 15 included studies are detailed in Table 1. The sample size ranged from 7 (Shamonki et al. 2016; Liu et al. 2017) to 70 (Hammond et al. 2017) couples undergoing assisted reproductive technology procedures. Gamete fertilization was performed mainly by intracytoplasmic sperm injection (ICSI) (86.6%, 13/15) (Stigliani et al. 2013; Wu et al. 2015; Xu et al. 2016; Shamonki et al. 2016; Liu et al. 2017; Hammond et al. 2017; Yang et al. 2017; Feichtinger et al. 2017; Vera-Rodriguez et al. 2018; Kuznyetsov et al. 2018; Li et al. 2018; Ho et al. 2018; Capalbo et al. 2018) and the other two studies did not report the fertilization method (Assou et al. 2014; Galluzzi et al. 2015). Cf-DNA from SCM was evaluated in 86.6% (13/15) of studies (Stigliani et al. 2013; Wu et al. 2015; Xu et al. 2016; Shamonki et al. 2016; Liu et al. 2017; Feichtinger et al. 2017; Vera-Rodriguez et al. 2018; Ho et al. 2018; Capalbo et al. 2018; Assou et al. 2014; Galluzzi et al. 2015), whereas a mixture of SCM and BF (SCM+BF) was assessed in 26.6% (4/15) of them (Hammond et al. 2017; Yang et al. 2017; Kuznyetsov et al. 2018; Li et al. 2018). The characteristics of the embryos associated to each SCM sample also were heterogeneous. Specifically, 93.3% (14/15) of studies evaluated cf-DNA released from fresh embryos (Stigliani et al. 2013; Galluzzi et al. 2015; Wu et al. 2015; Xu et al. 2016; Shamonki et al. 2016; Liu et al. 2017; Hammond et al.

2017; Yang et al. 2017; Feichtinger et al. 2017; Vera-Rodriguez et al. 2018; Kuznyetsov et al. 2018; Li et al. 2018; Ho et al. 2018; Capalbo et al. 2018), and 20.0% (3/15) of studies (Xu et al. 2016; Kuznyetsov et al. 2018; Ho et al. 2018) assessed cf-DNA secreted from frozen/thawed embryos. Moreover, embryo micromanipulation (e.g., assisted hatching (AH), blastocoel collapse (BC) or embryo biopsy) was performed before SCM sampling in 60.0% (9/15) of studies (Wu et al. 2015; Shamonki et al. 2016; Hammond et al. 2017; Yang et al. 2017; Feichtinger et al. 2017; Vera-Rodriguez et al. 2018; Kuznyetsov et al. 2018; Li et al. 2018; Ho et al. 2018). The release of cf-DNA in SCM was evaluated throughout early development: zygotes (D1) (Galluzzi et al. 2015; Yang et al. 2017; Ho et al. 2018), cleaved embryos (D2/D3) (Stigliani et al. 2013; Assou et al. 2014; Galluzzi et al. 2015; Wu et al. 2015; Xu et al. 2016; Shamonki et al. 2016; Hammond et al. 2017; Yang et al. 2017; Vera-Rodriguez et al. 2018; Kuznyetsov et al. 2018; Li et al. 2018; Ho et al. 2018), morula-stage embryos (D4) (Yang et al. 2017; Wu et al. 2015), and blastocysts (D5-D6) (Assou et al. 2014; Galluzzi et al. 2015; Wu et al. 2015; Xu et al. 2016; Shamonki et al. 2016; Liu et al. 2017; Hammond et al. 2017; Yang et al. 2017; Feichtinger et al. 2017; Vera-Rodriguez et al. 2018; Kuznyetsov et al. 2018; Li et al. 2018; Ho et al. 2018; Capalbo et al. 2018). The SCM-embryo incubation time varied from 1 day (Stigliani et al. 2013; Wu et al. 2015; Yang et al. 2017; Kuznyetsov et al. 2018) to 5 days (Liu et al. 2017; Hammond et al. 2017; Feichtinger et al. 2017; Capalbo et al. 2018).

Cf-DNA detection and quantification in SCM

For the first objective, fourteen studies that investigated cf-DNA detection rate or quantification in SCM were included (Table 2). Their mNOS score, to assess the study quality, varied from 2/12 to 12/12 (mean 8.1/12) (Supplementary Table 1). The mNOS score

was higher than 6 in 78.6% (11/14) of studies, and one study obtained the highest score (i.e., 12/12) (Kuznyetsov et al. 2018).

In SCM, nDNA detection rate varied from 6.7% (Galluzzi et al. 2015) to 100% (Assou et al. 2014; Yang et al. 2017; Xu et al. 2016; Shamonki et al. 2016; Kuznyetsov et al. 2018; Ho et al. 2018) (Table 2). The lowest nDNA detection rate (i.e., 6.7%) was reported by Galluzzi et al. (mNOS score = 5/12) who used quantitative PCR to detect the multi-copy *TSPY1* gene (located on chromosome Y) in SCM samples of male and female embryos on D3 (Galluzzi et al. 2015). Conversely, the study with the highest mNOS score reported a nDNA detection rate of 100% in SCM (Kuznyetsov et al. 2018). The detection rate of mtDNA was stated only in one study (98.8%) (Stigliani et al. 2013)). Concerning the quantification of cf-DNA in SCM, higher amounts of mtDNA than nDNA have been reported in the same SCM samples (Hammond et al. 2017), with high variability in the mtDNA/nDNA copy number ratio, ranging from 5 to 5221 (average: 394) (Stigliani et al. 2013).

The detection/amplification rates or amount of nDNA varied among studies, suggesting that cf-DNA release is influenced by some clinical, biological or technical parameters. Specifically, cf-DNA detection rates in SCM were higher after DNA amplification (62.5-100%) (i.e., 80-100% after Multiple Annealing and Looping Based Amplification Cycles (MALBAC) (Xu et al. 2016; Liu et al. 2017; Li et al. 2018), 62.5-100% after MALBAC-like (Galluzzi et al. 2015; Feichtinger et al. 2017; Vera-Rodriguez et al. 2018; Kuznyetsov et al. 2018; Ho et al. 2018), 96.5-100% after Multiple Displacement Amplification (MDA)(Shamonki et al. 2016), and 62.9% after Polymerase Chain Reaction (PCR) (Stigliani et al. 2013)) compared with the results obtained without DNA amplification (6.7-100%)(Galluzzi et al. 2015; Vera-Rodriguez et al. 2018; Hammond et al. 2017; Wu et al. 2015; Yang et al. 2017; Assou et al. 2014). A significant increase in the detection/amplification rates or in the total amount of nDNA was also observed in SCM from

blastocysts compared with cleaved embryos (Yang et al. 2017; Wu et al. 2015; Assou et al. 2014; Galluzzi et al. 2015; Hammond et al. 2017). Moreover, a short incubation time (<24h) between SCM and embryo was considered suboptimal for nDNA detection (Kuznyetsov et al. 2018). Conversely, a long incubation time (>48-72h) seems to compromise nDNA integrity (Ho et al. 2018) while increasing its quantity (Hammond et al. 2017). The clinical and biological parameters influencing mtDNA detection rate have not been investigated yet.

Embryo vitrification (Kuznyetsov et al. 2018), embryo ploidy (Ho et al. 2018; Vera-Rodriguez et al. 2018) and embryo sex (Vera-Rodriguez et al. 2018) do not seem to significantly influence cf-DNA release in SCM. The results on the association between embryo fragmentation and cf-DNA release in SCM are conflicting. One study reported an increase of cf-DNA in SCM associated with fragmented embryos (Stigliani et al. 2013), whereas another study did not find any significant association (Ho et al. 2018). Moreover, cf-DNA detection was not modified by artificial opening of the zona pellucida due to AH (Ho et al. 2018). Likewise, the presence of some fragments or cells outside the zona pellucida (e.g., hatching/hatched versus enclosed blastocysts) did not seem to influence cf-DNA release in SCM (Vera-Rodriguez et al. 2018). BC also did not increase cf-DNA detection rate in SCM+BF samples (Yang et al. 2017; Kuznyetsov et al. 2018; Li et al. 2018) compared with SCM alone (Shamonki et al. 2016; Liu et al. 2017; Feichtinger et al. 2017; Ho et al. 2018; Vera-Rodriguez et al. 2018). DNA extraction from SCM did not improve cf-DNA detection and amplification rates (Stigliani et al. 2013; Capalbo et al. 2018). The double amplification strategy proposed by *Vera-Rodriguez et al* does not seem to reduce the amplification failure rates (Vera-Rodriguez et al. 2018).

Exogenous cf-DNA was detected in unused IVF medium, additives (e.g., human albumin) (Liu et al. 2017; Hammond et al. 2017; Vera-Rodriguez et al. 2018), and embryo-free medium droplets (Wu et al. 2015; Hammond et al. 2017; Vera-Rodriguez et al. 2018), highlighting the

risk of exogenous DNA contamination before, during, and after culture. In total, 57.1% (8/14) of studies evaluated the presence of cf-DNA in embryo-free droplets of cultured medium (Kuznyetsov et al. 2018; Li et al. 2018; Galluzzi et al. 2015; Xu et al. 2016; Ho et al. 2018; Capalbo et al. 2018; Vera-Rodriguez et al. 2018; Hammond et al. 2017), and 25% (2/8) of them reported the presence of DNA contamination (Vera-Rodriguez et al. 2018; Hammond et al. 2017). Vera-Rodriguez et al. found a median cf-DNA amount of 1.4 pg (interquartile range: 1.0–4.4 pg) in embryo-free droplets of cultured medium before DNA amplification (Vera-Rodriguez et al. 2018). Cf-DNA was detected in all negative controls (100% (17/17) (Vera-Rodriguez et al. 2018). Hammond et al. reported a mean number of 15 ± 4 copies of mtDNA and one copy of nDNA in embryo-free droplets of cultured medium without DNA amplification (Hammond et al. 2017). Similar low levels of mtDNA and nDNA were detected in unused IVF medium and additives (Hammond et al. 2017). In studies that reported DNA contamination in embryo-free droplets of cultured medium, the amount of cf-DNA was significantly lower than in SCM (Vera-Rodriguez et al. 2018; Hammond et al. 2017). In studies with mNOS higher than 6/12 that did not find cf-DNA in embryo-free droplets of protein-supplemented culture medium, cf-DNA detection or amplification rates ranged from 97.5% (Li et al. 2018) to 100% (Xu et al. 2016; Kuznyetsov et al. 2018) (Li et al. 2018; Xu et al. 2016; Kuznyetsov et al. 2018; Ho et al. 2018), and the mean concentration of amplified cf-DNA varied from 15.2 ± 2.7 ng/µl (Kuznyetsov et al. 2018) to 58.03 ± 35.87 ng/µl (Li et al. 2018) in SCM after 24-48h of incubation with blastocysts.

SCM-PGT reliability

For the second objective, 10 studies that evaluated SCM-PGT reliability were reviewed (Table 3). Their mean mNOS score was 9.6 (range: 6-12), and two studies obtained the highest score (12/12) (Kuznyetsov et al. 2018; Xu et al. 2016)(Supplementary Table 2).

For genetic DNA analysis, 60% (6/10) of studies used Next Generation Sequencing (NGS), 20% (2/10) used array Comparative Genomic Hybridization (aCGH), and 20% (2/10) quantitative PCR (qPCR).

The genetic results obtained using SCM-PGT were compared with those obtained using invasive techniques (polar bodies (Feichtinger et al. 2017), embryo cells by blastomere/trophectoderm biopsy (Galluzzi et al. 2015; Shamonki et al. 2016; Liu et al. 2017; Vera-Rodriguez et al. 2018; Kuznyetsov et al. 2018; Li et al. 2018; Ho et al. 2018; Capalbo et al. 2018), and whole embryos (Xu et al. 2016; Kuznyetsov et al. 2018; Li et al. 2018; Ho et al. 2018)), and also with clinical outcomes (pregnancy (Galluzzi et al. 2015), and live birth (Xu et al. 2016)). Whole embryo as DNA template, pregnancy, and live birth were considered the optimal standard for genetic result comparisons. Indeed, discordant results were reported between whole embryo and trophectoderm biopsy analyses for the same embryo, although sampling was performed at the same time (Kuznyetsov et al. 2018; Li et al. 2018; Vera-Rodriguez et al. 2018; Feichtinger et al. 2017). These discrepancies varied from 7.4% (2/27) (Ho et al. 2018) to 18.4% (7/38) (Li et al. 2018). To determine the causes of these discrepancy, *Vera-Rodriguez et al.* assessed the chromosomal status of single cells in whole-embryo analyses and found that 91.7% (11/12) of the analysed blastocysts displayed a mosaic pattern (i.e., ≥2 cell populations with different genotypes in the same embryo) for chromosomal ploidy (Vera-Rodriguez et al. 2018). Genetic discordance between inner cell mass and trophectoderm cells also was reported (Vera-Rodriguez and Rubio 2017), indicating that comparison with whole-embryo data is preferable for evaluating SCM-PGT reliability. However, only 40% (4/10) of the included studies used whole embryos as DNA reference (Xu et al. 2016; Kuznyetsov et al. 2018; Li et al. 2018; Ho et al. 2018).

Concordance rates were expressed as general concordance (i.e., healthy versus unhealthy) or full concordance (i.e., specific number and type of genetic alterations). Overall,

general and full concordance rates were hugely heterogeneous among studies. The general concordance rates varied from 18.2% (Ho et al. 2018) to 100% (Galluzzi et al. 2015; Xu et al. 2016; Shamonki et al. 2016; Kuznyetsov et al. 2018). The lowest general concordance rate (i.e., 18.2%) was reported for SCM of arrested embryos (Ho et al. 2018). One study evaluated the general concordance rate of the genetic analyses between SCM and PB biopsy (72.2%) (Feichtinger et al. 2017). Seven studies reported the general concordance rate between SCM and embryo/trophectoderm biopsy genetic analyses that ranged from 50% (Galluzzi et al. 2015) to 100% (Galluzzi et al. 2015; Shamonki et al. 2016; Kuznyetsov et al. 2018). The general concordance rates for results obtained by SCM and whole-embryo analysis ranged from 65.0% (13/20) (Ho et al. 2018) to 100% (3/3) (Xu et al. 2016). The two studies with the highest mNOS score reported high general concordance rates (85.7% and 100%) for results obtained using SCM incubated with growing blastocysts for 24-48h and whole embryo (Xu et al. 2016; Kuznyetsov et al. 2018).

Overall, the full concordance rates were lower compared with the general concordance rates in the included studies (Liu et al. 2017; Xu et al. 2016; Feichtinger et al. 2017; Vera-Rodriguez et al. 2018; Kuznyetsov et al. 2018; Li et al. 2018), and varied from 5.9% (Vera-Rodriguez et al. 2018) to 100% (Galluzzi et al. 2015; Xu et al. 2016; Shamonki et al. 2016). The full concordance rates between SCM incubated with growing blastocysts for 24-48h and whole embryo DNA ranged from 57.1% to 100% in the two studies with the highest mNOS score (Xu et al. 2016; Kuznyetsov et al. 2018). Discrepancies concerned the genetic alteration patterns detected in unhealthy embryos: complete versus mosaic aneuploidy, complementary aneuploidies in term of loss versus gain of chromosomes, a mixture of shared and unshared aneuploidies, and aneuploidy but for different chromosomes (Vera-Rodriguez et al. 2018; Xu et al. 2016; Liu et al. 2017; Feichtinger et al. 2017; Kuznyetsov et al. 2018; Li et al. 2018). SCM-PGT allowed detecting unbalanced translocations and mosaicisms (Kuznyetsov et al.

2018; Li et al. 2018). Two studies reported five healthy pregnancies (Galluzzi et al. 2015) and six live births of healthy children based on the SCM genetic analyses (Xu et al. 2016), with general concordance rates of 100% between SCM and pregnancies or live births (Xu et al. 2016; Galluzzi et al. 2015).

Three studies used single nucleotide polymorphism (SNP) or microsatellite analysis to evaluate SCM contamination (full or partial) by extra-embryonic DNA and highlighted that exogenous/maternal DNA in SCM is a major risk factor for genetic misdiagnosis (Vera-Rodriguez et al. 2018; Capalbo et al. 2018; Liu et al. 2017). Extra-embryonic cf-DNA may result from exogenous DNA present in the IVF medium (Liu et al. 2017; Hammond et al. 2017; Vera-Rodriguez et al. 2018; Wu et al. 2015) or from maternal DNA (probably originating from cumulus/corona radiata cells) (Feichtinger et al. 2017; Vera-Rodriguez et al. 2018; Capalbo et al. 2018). In studies that investigated the presence of extra-embryonic DNA in SCM, exogenous and maternal DNA were detected in approximatively 25% and 50% of SCM samples, respectively (Vera-Rodriguez et al. 2018; Liu et al. 2017). The proportion of extra-embryonic cf-DNA in SCM varied from 0% (Wu et al. 2015; Shamonki et al. 2016; Yang et al. 2017; Kuznyetsov et al. 2018; Li et al. 2018) to 100% (Vera-Rodriguez et al. 2018). *Vera-Rodriguez et al.* estimated that the median percentage of embryonic DNA was 8% (min-max 0-100%) (Vera-Rodriguez et al. 2018). The percentage of embryonic DNA was higher in concordant samples (i.e., 14%) than in maternally-contaminated samples (i.e., 6%), but this difference was not statistically significant (Vera-Rodriguez et al. 2018). Interestingly, the ratio of embryonic cf-DNA to maternal cf-DNA increased at later stages (Vera-Rodriguez et al. 2018; Galluzzi et al. 2015). Allele Drop-Out (ADO) was observed more frequently for paternal than maternal alleles for successfully amplified loci, suggesting that the level of paternal DNA is lower than that of maternal DNA in the case of SBM contamination (Capalbo et al. 2018).

The embryo developmental stage and SCM collection timing influence the feasibility and reliability of genetic analyses (Capalbo et al. 2018; Ho et al. 2018). The probability of generating sufficient sequence reads to determine the chromosome copy status and the concordance rate were significantly increased in SCM samples from blastocysts compared with D5 arrested embryos (60.6% versus 39.4% and 65.0% versus 18.2%, respectively) (Ho et al. 2018). The impact of embryo stage on the general and full concordance rates is unclear. Only two studies reported the concordance rates at the cleavage stage (Ho et al. 2018; Galluzzi et al. 2015). Galluzzi et al. found that the general and full concordance rates for the TPSY1 gene between TE biopsy and SCM were higher when using SCM associated with blastocysts (100%) than cleaved embryos (50%) (Galluzzi et al. 2015). Ho et al. reported a similar general concordance rate (56.3% from SCM associated with cleaved embryos and 45.5% from SCM associated with blastocysts) with lower amplification rate when using SCM associated with cleaved embryos (40% and 82.5% respectively) (Ho et al. 2018). The increase in embryonic DNA in SCM at later stages may contribute to the higher concordance rates observed with SCM from blastocysts. Conversely, DNA extraction before amplification (Capalbo et al. 2018) and sequence depth (Ho et al. 2018), AH (Ho et al. 2018), as well as morphological scores and fragmentation rate (Ho et al. 2018) do not seem to influence SCM-PGT reliability.

DISCUSSION

One of the biggest challenges in the field of PGT is to validate a non-invasive procedure for the genetic diagnosis of *in vitro* developing embryos (Kuliev and Rechitsky 2017; Farra, Choucair, and Awwad 2018; Munne 2018) with the aim of improving PGT cost-efficiency and safety (Figure 3). SCM collection is an easy procedure that can be safely performed for all embryos. Indeed, SCM collection does not require special expertise in embryo manipulation compared with embryo biopsy/blastocentesis, thus reducing the laboratory workload. Moreover, SCM collection is non-invasive. By avoiding all detrimental effects of suboptimal micromanipulations and/or potential risks due to invasive procedures, it does not affect the embryo development and reproductive potential. Moreover, SCM can be collected at any pre-implantation developmental stage; even cleaved embryos with less than 6 cells at D3 and early blastocysts can be tested, unlike iPGT that is based on embryo biopsy or blastocentesis. Hence, SCM-PGT might be particularly suitable for cultured growing embryos with low implantation potential that cannot be tested by iPGT. SCM-PGT is relatively fast (<12 hours from SCM collection to genetic analysis) (Wu et al. 2015; Li et al. 2018; Xu et al. 2016), and results may be available before embryo transfer or freezing. In the case of positive diagnosis, another SCM sample should be collected after 24h of incubation for confirmation

Altogether, the published data demonstrated that SCM is a potential alternative source of embryo DNA and a promising procedure for the genetic testing of all developing embryos. However, detection/amplification and concordance rates need to be improved before implementing SCM-PGT in routine clinical practice. Moreover, SCM- PGT standardization and the establishment of guidelines are mandatory to allow the reliable comparison of outcomes and the verification of result consistency among IVF centres.

Design of the studies

The studies included in this systematic review have several limitations (Figure 3). First, their limited number (n=15 included studies) and the small sample size of some studies (Assou et al. 2014; Yang et al. 2017; Galluzzi et al. 2015; Shamonki et al. 2016) weaken the result strength and interpretation. Second, they display considerable differences in design, studied population, and methods. The SCM clinical and biological characteristics (e.g., SCM/SCM+BF, fresh/frozen embryos, blastocysts/cleaved embryos, short/long incubation time) as well as the genetic approaches (e.g., +/- DNA amplification, genetic testing technology) are heterogeneous among studies, thus limiting the result comparability. Third, data that are fully or partially based on poorly developing or arrested embryos (Stigliani et al. 2013; Wu et al. 2015; Liu et al. 2017; Feichtinger et al. 2017; Ho et al. 2018; Li et al. 2018) have a limited interest because such embryos are not selected for transfer or cryopreservation due to their low implantation potential. Moreover, they seem to present higher amplification failure (Liu et al. 2017) and more chromosome variations, increasing the complexity of the genetic analysis and decreasing the concordance rates (Ho et al. 2018). As the results obtained with poor developing embryos cannot be generalized to embryos with good potential, they are not suitable for assessing SCM-PGT reliability. Fourth, the inability to account for extra-embryonic (exogenous or maternal) DNA contamination in most studies strongly affects the interpretation of cf-DNA detection, amplification and concordance rates. The presence of maternal DNA in SCM (Vera-Rodriguez et al. 2018; Hammond et al. 2017; Feichtinger et al. 2017), despite oocyte denudation and extensive washing, requires a genetic technique that allows discriminating between maternal and embryonic DNA to prevent non-embryonic DNA interference. As cf-DNA concentration is quite low in SCM, any contamination by exogenous and/or maternal DNA will misleadingly increase cf-DNA detection rate, while decreasing SCM-PGT diagnostic accuracy.

Detection, amplification and quantification of cf-DNA from SCM

All selected studies demonstrated the presence of cf-DNA in SCM of human embryos in culture. Its detection rate was generally high in SCM samples, suggesting that cf-DNA is frequently released during early embryo development *in vitro*. The discrepancies in the detection/amplification rates and in the total amount of cf-DNA obtained from SCM might be partially explained by the heterogeneous clinical and biological characteristics of SCM among studies. First, in some studies, the entire SCM droplet was not collected (Galluzzi et al. 2015; Xu et al. 2016; Yang et al. 2017; Feichtinger et al. 2017; Vera-Rodriguez et al. 2018; Ho et al. 2018). This might have contributed to decreasing the amount of cf-DNA and the detection/amplification rates, and might have affected the cf-DNA concentration homogeneity in the tested SCM samples. Second, the SCM collection timing significantly influenced cf-DNA release in SCM, suggesting that both embryo stage and incubation time represent critical factors in SCM-PGT. Indeed, cf-DNA was not detected in SCM from zygotes (Yang et al. 2017), suggesting that cf-DNA release could start at the time of blastomere division. Conversely, it was detected in SCM from cleaved embryos to blastocysts, indicating that it is released throughout early embryonic development. However, the detection/amplification rates and the total amount of cf-DNA were higher for SCM from blastocysts compared with cleaved embryos (Galluzzi et al. 2015; Wu et al. 2015; Hammond et al. 2017; Yang et al. 2017). Concerning the incubation time, a contact of 24-48h between SCM and embryo seems adequate to optimize cf-DNA release in the medium (Hammond et al. 2017), while preventing cf-DNA degradation (Ho et al. 2018; Kuznyetsov et al. 2018). Conversely, the use of thawed embryos after vitrification does not seem to affect cf-DNA release. Likewise, embryo manipulation (e.g., AH and BC) before SCM collection does not increase cf-DNA release (Ho et al. 2018; Kuznyetsov et al. 2018; Li et al. 2018). The absence of BC effect on cf-DNA

release is in agreement with the higher amount of cf-DNA quantified in SCM than in BF before (≈ 10 pg/µl in BF (Palini et al. 2013) and ≈ 50 pg/µl in SCM (Wu et al. 2015)) and after DNA amplification (10 ng/µl in BF and 55 ng/µl in SCM (Li et al. 2018)). Moreover, the locus amplification rate was significantly higher when using cf-DNA from SCM than from BF for the same embryo (Capalbo et al. 2018). Furthermore, the genetic results obtained with cf-DNA from SCM were more frequently concordant with those of TE biopsies compared with cf-DNA from BF samples (Capalbo et al. 2018). Altogether, these data suggest that combining SCM with BF does not increase SCM-PGT efficiency.

The technology used to detect and quantify cf-DNA in SCM also might influence the results. Li et al. performed DNA quantification using spectrophotometry (i.e., NanoDrop) (Li et al. 2018) that measures double stranded DNA but also other nucleic acids (e.g., RNA, single-stranded DNA, free nucleotides) (Garcia-Elias et al. 2017), thus potentially overestimating DNA concentration. Methods based on fluorescent dyes (e.g., Qubit) (Shamonki et al. 2016; Feichtinger et al. 2017; Ho et al. 2018; Vera-Rodriguez et al. 2018; Kuznyetsov et al. 2018) only measure double-stranded DNA, but do not differentiate between DNA molecules of different lengths, thus potentially resulting in the quantification of primer dimers, adapter dimers, and other fragments without adapters. Conversely, electrophoresis-based methods (e.g., Bioanalyzer) (Feichtinger et al. 2017; Liu et al. 2017; Vera-Rodriguez et al. 2018) allow the visual assessment of the amplicon quality, and consequently the exclusion of small molecules, such as primer and adapter dimers. For instance, qPCR is considered the most sensitive approach for different applications (Klein 2002). However, its sensitivity is influenced by numerous factors, particularly the number of copies of the targeted locus, the presence of degraded nucleic fragments that may not be amplified, and the sample purity (contaminants might act as amplification inhibitors). This last point is critical because the IVF

medium components could have inhibitory effects on PCR amplifications (Galluzzi et al. 2015).

The presence of extra-embryonic cf-DNA in SCM also falsely increases cf-DNA detection/amplification rates or quantity. Seven studies did not evaluate cf-DNA presence in embryo-free droplets of protein-supplemented culture medium (Stigliani et al. 2013; Assou et al. 2014; Wu et al. 2015; Shamonki et al. 2016; Liu et al. 2017; Yang et al. 2017; Feichtinger et al. 2017), limiting the accuracy of their results. In studies that confirmed the absence of cf-DNA in embryo-free droplets of cultured medium and with a mNOS higher than 6/12 (Li et al. 2018; Xu et al. 2016; Kuznyetsov et al. 2018; Ho et al. 2018), detection or amplification failure ranged from 0% (Xu et al. 2016; Kuznyetsov et al. 2018) to 2.5% (Li et al. 2018), which is presently considered acceptable in iPGT (Handyside 2016; Cimadomo et al. 2018).

SCM PGT reliability

Although cf-DNA from SCM has been successfully detected and amplified, the reliability of this alternative source of genetic material for embryo assessment is still debated. Discrepancies concerning the embryo genetic status concordance rates obtained with SCM and other DNA sources (e.g., polar bodies, embryo/trophectoderm biopsies, whole embryos) have been reported. This can be explained by a number of possibilities (Figure 3).

First, the low amount of cf-DNA in SCM is associated with genetic analysis pitfalls. Therefore, whole genome amplification (WGA) is usually performed to increase the amount of DNA prior to genetic analyses. The current WGA approaches are based on three different strategies: MDA, PCR-based methods, and a combination of both (e.g., MALBAC) (Hou et al. 2015). The genomic coverage level, amplification bias in function of the GC content, ADO prevalence, preferential allele amplifications, chimeric DNA-molecules, and nucleotide copy errors can vary significantly among WGA approaches, making some methods better suited

than others for detecting specific genetic variation classes (Macaulay and Voet 2014). PCR-based WGA methods show the best reproducibility and accuracy for copy number variation (CNV) detection, despite their limited mapping efficiency and genome recovery. Conversely, MDA and MALBAC can accurately detect single nucleotide variations (SNV) with higher efficiency and sensitivity than WGA PCR-based methods due to the higher fidelity of DNA polymerase and the higher genomic coverage level (Hou et al. 2015; Macaulay and Voet 2014). Therefore, PCR-based methods may be especially suited for CNV analysis and for PGT-A, while MDA or WGA hybrid approaches are more appropriate for SNV analysis (de Bourcy et al. 2014). Although DNA amplification could theoretically lead to a high error rate due to amplification bias (Ho et al. 2018), this risk is typically minimized during targeted NGS due to its high coverage for consensus sequences that reduces the noise from random errors. New amplification techniques that minimize the variation coefficients and reduce the amount of DNA template are needed for SCM-PGT clinical implementation. WGA methods with improved CNV accuracy and resolution are currently tested (Chen et al. 2017; Tan et al. 2018). The development of genetic techniques with lower detection limits (e.g., pyrosequencing and digital PCR) also could improve SCM-PGT diagnostic efficiency.

Second, the source of the reference DNA (i.e., polar bodies, embryo cells or whole embryos) also could influence the concordance rates. Indeed, polar body DNA mirrors the genetic status of the corresponding oocyte. Therefore, it will allow detecting genetic abnormalities of maternal origin, but not assessing the paternal contribution and the subsequent genetic events in embryonic cells. Yet, mitotic errors occur frequently during preimplantation development, leading to a high risk of mosaicism in human embryos (from 13% to 73%, mean ≈ 30%) (Munne et al. 2017; van Echten-Arends et al. 2011; Fragouli et al. 2011; Ruttanajit et al. 2016; Capalbo, Wright, et al. 2013; Li et al. 2018). As embryo/trophectoderm biopsies rely on the random retrieval of 1-10 cells within the growing

embryo, they may not reflect the genetic status of the other cells in a mosaic embryo. Hence, the use of polar body and embryo/trophectoderm biopsies for the reference DNA could misleadingly contributes to the low concordance rates. This suggests that whole-embryo analysis is the best approach to determine SCM-PGT reliability. Miscarriage rates, invasive prenatal diagnostic screening or live births should also be reported to confirm SCM-PGT accuracy and safety. In the case of genetic misdiagnosis, the transfer of false-negative embryos usually does not result in successful pregnancies (Xu et al. 2016). Otherwise, they could be detected by non-invasive prenatal testing (Xu et al. 2016). False positive results can be further verified by blastocyst biopsy (Xu et al. 2016). Results were fully concordant when SCM-PGT was compared with pregnancies (Galluzzi et al. 2015) or live births (Xu et al. 2016). Moreover, a recent study reported 27 healthy births following SCM-PGT (Fang et al. 2019). Although encouraging, these results do not allow reaching any conclusion because the number of cases is too low.

Third, discordance may also be attributed to partial or full SCM contamination by exogenous (Liu et al. 2017; Hammond et al. 2017; Vera-Rodriguez et al. 2018; Wu et al. 2015) or maternal DNA despite the careful removal of cumulus cells during oocyte denudation prior to ICSI (Hammond et al. 2017; Vera-Rodriguez et al. 2018). Therefore, SCM-PGT requires strategies to discriminate between exogenous/maternal DNA and embryonic DNA in SCM to improve the general and full concordance rates, but this was done only in 30% (3/10) of studies (Liu et al. 2017; Vera-Rodriguez et al. 2018; Capalbo et al. 2018). Embryonic cf-DNA differs in size and contains different preferred ends than maternal fragments (Peng and Jiang 2017; Chan et al. 2016). These features could be used to develop new approaches that will allow discriminating embryonic cf-DNA from exogenous DNA in SCM in order to improve SCM-PGT sensitivity and specificity.

Fourth, cf-DNA origin also could explain the discrepant results. Indeed, one major concern is whether cf-DNA from SCM is representative of the whole genome status of an embryo. It is currently thought that nDNA is released by degenerated organelles and cells (Stigliani et al. 2013; Xu et al. 2016; Hammond et al. 2017; Zhang, Li, et al. 2016; D'Alessandro et al. 2012; Jensen et al. 2013). This hypothesis is supported by (1) the low molecular weight of cf-DNA (from 100 to 1000 bp, mean ≈ 400 bp (Stigliani et al. 2013) compared with the 3 million bp of genomic DNA), and (2) the high rate of complementary and divergent aneuploidies between SCM and TE biopsy or whole embryo analysis (Vera-Rodriguez and Rubio 2017). Therefore, cf-DNA detected in SCM should mostly reflect the genetic constitution of discarded cells, potentially diverging from the growing embryo genetic status. Cell apoptosis is frequent in the inner cell mass (ICM) and trophectoderm during embryo development (Hardy 1997, Hardy 1999), suggesting that SCM contains DNA from both cell lineages. Moreover, the number of apoptotic cells progressively increases with the total number of embryonic cells (Hardy, Handyside, and Winston 1989). Bolton et al. showed that in the mouse, ICM cells (euploid and aneuploid) undergo more frequently apoptosis than TE cells (Bolton et al. 2016). This suggests that DNA from SCM could preferentially reflect the genetic status of the ICM than of the TE, whereas DNA in TE biopsies only reflects the TE genetic status. Therefore, SCM-PGT could be more informative than TE biopsy. The similar amount of cf-DNA in SCM from aneuploid and euploid embryos suggests that both cell types undergo apoptosis during embryo development (Vera-Rodriguez et al. 2018). In some cases, aneuploid cells are extensively eliminated from the growing embryo, leading to embryo self-correction (Taylor et al. 2014; Bazrgar et al. 2013; van Echten-Arends et al. 2011; Capalbo, Bono, et al. 2013; Sachdev et al. 2017; Greco, Minasi, and Fiorentino 2015; Zore et al. 2019). The finding that euploid embryos and healthy live births are obtained after the transfer of mosaic embryos supports the hypothesis of genetic self-correction by the

embryo (Sachdev et al. 2017; Bazrgar et al. 2013; Greco, Minasi, and Fiorentino 2015; Zhang et al. 2019). In this case, the genetic profile of nDNA generated from discarded aneuploid cells could be inconsistent with that of the growing embryo (specifically in the ICM), leading to false-positive genetic results and misdiagnosis of embryos that underwent rescue mechanisms. Moreover, one could hypothesize that top quality embryos may be unsuitable for SCM-PGT due to insufficient apoptosis. Contrary to nDNA, mtDNA may originate from the loss of cytoplasm from actively dividing blastomeres and also from degenerated cells of the whole embryo, thus reflecting the average of all genetic alterations that occurred throughout embryo development (Stigliani et al. 2013; Hammond et al. 2017). Future clinical studies should determine the origin of both SCM nDNA and mtDNA to conclude on whether cf-DNA really reflects the embryo genetic status. In that case, SCM PGT could increase the diagnosis of unhealthy embryos by improving the detection of mosaic embryos compared to iPGT.

These four limitations limit any conclusion on SCM-PGT diagnostic efficiency and clinical efficacy.

Identification of optimal procedures

The identification of a standardized protocol for SCM-PGT represents a significant short-term goal. Indeed, improvements in DNA collection methods, DNA amplification, and screening techniques are necessary to determine the reliability of SCM-PGT. The heterogeneity in the design of the selected studies led to the identification of many factors that might affect cf-DNA detection and amplification rates, as well as SCM-PGT reliability. Their impact still needs to be extensively evaluated in future studies before establishing a standardized procedure for SCM-PGT. On the basis of the available data, we propose the following measures to increase the detection of embryonic DNA and to reduce the risk of

exogenous/maternal DNA contamination, in order to improve SCM-PGT reliability for futures clinical studies.

1. Procedure

To prevent contamination by extra-embryonic cf-DNA, all operators should wear gloves, masks, caps and laboratory coats during the procedure. Physical isolation should be guaranteed by working in laminar flow hoods. Disposable sterile pipettes, tubes and tips should be used for each embryo or SCM sample. All procedures (e.g., gamete and embryo handling, SCM collection, and subsequent analysis) should be performed using RNase-DNase-free PCR-clean LoRetention two-phase filter tips and RNase-DNase-free PCR-clean LoRetention PCR tubes to protect against contamination by aerosol and biomolecules.

2. Gamete fertilization and embryo culture

    a. Gamete fertilization

We recommend using ICSI for gamete fertilization to minimize SCM contamination by parental DNA (from cumulus cells and/or spermatozoa). After removal of all visible cumulus and corona radiata cells, oocytes should be carefully washed and transferred into new IVF medium. Although the benefit-risk balance seems to favour the use of ICSI in future studies on SCM-PGT, the impact of conventional IVF on cf-DNA release still need to be determined because the systematic use of ICSI raises some concerns (Rubino et al. 2016; Crawford and Ledger 2019; Palermo, Neri, and Rosenwaks 2015).

    b. Embryo culture

SCM-PGT requires individual embryo culture in separate medium droplets. We propose to collect SCM from D3 to D5 embryos for four reasons: i) D3 embryos can be extensively washed and the IVF medium changed before the start of the incubation for SCM collection, thus decreasing the risk of maternal contamination due to residual corona radiata/cumulus cells; ii) SCM associated with blastocysts displays higher cf-DNA detection and amplification

rates and higher proportions of embryo cf-DNA compared with earlier stages; iii) the incubation time should be between 24h and 48h to optimize cf-DNA release, while preventing its degradation; iv) blastocysts are associated with higher implantation potential, improving pregnancy rates while decreasing both PGT workload and cost, and present a lower risk of mosaicism compared with cleavage embryos (McCoy 2017; Vera-Rodriguez and Rubio 2017).

c. Negative controls: unused and embryo-free medium droplets

Negative controls, such as unused IVF medium and additives (e.g., protein supplement), should be directly collected and stored for DNA contamination testing (Shamonki et al. 2016; Li et al. 2018; Capalbo et al. 2018). Moreover, embryo-free droplets of medium incubated in the same dish should also be processed as negative controls (Galluzzi et al. 2015; Wu et al. 2015; Liu et al. 2017; Shamonki et al. 2016; Kuznyetsov et al. 2018; Li et al. 2018; Ho et al. 2018; Capalbo et al. 2018).

3. SCM collection and storage

We recommend collecting the full volume of SCM to maximize the cf-DNA amount obtained from one sample and to increase the detection and amplification rates, while minimizing the risk of cf-DNA concentration heterogeneity among SCM samples. Indeed, cf-DNA repartition in SCM is currently unknown. All samples should be immediately frozen and kept at -20°C for short-term storage (few days before testing) or at -80°C for long-term storage (months to years).

4. DNA amplification

The limited concentration of embryo DNA could limit the determination of the embryo genetic status by direct SCM analysis, particularly by whole-genome sequencing. Therefore, we recommend cf-DNA amplification (without prior DNA purification) with high uniformity and fidelity for SCM-PGT. Double DNA amplification does not seem to be needed to

decrease the amplification failure, and therefore we do not recommend it. The DNA amplification step should include also negative controls, such as unused IVF medium (+/- protein additives), embryo-free droplets, and no template (RNase/DNase-free water), to exclude accidental contamination before or during embryo culture and during SCM collection/handling.

5. Genetic testing

We recommend to use whole embryo DNA as reference source DNA to evaluate SCM-PGT reliability. The components present in the IVF medium could have inhibitory effects on PCR reactions, with a delay in the Ct values without affecting the reaction efficiencies (Galluzzi et al. 2015). Therefore, we recommend to generate standard curves with an adequate volume of IVF medium to take into account these inhibitory effects when PCR-based methods are used. Genetic testing should be performed with amplified negative controls in each run as well as with no template controls (RNase/DNase-free water) to exclude accidental contamination during genetic testing. Moreover, SNP or microsatellite analysis should be performed to exclude extra-embryonic contamination by exogenous/maternal DNA. Indeed, SNPs observed in SCM DNA and in embryo DNA (from blastomere/trophectoderm biopsies or whole embryos) but not in maternal DNA (e.g., from follicular fluid or granulosa/cumulus cells) will confirm the embryonic origin of the cf-DNA detected in the SCM sample, supporting the concordance between SCM and embryo genetic analyses. Only SNPs on autosomes should be considered because the Y chromosome could be absent in SCM. These additional tests decrease the overall cost-efficiency of non-invasive PGT, but are absolutely necessary. NGS technologies are the most promising approach for non-invasive PGT-A (Rubio et al. 2019). It has been reported that NGS can detect balanced translocation in SCM (Kuznyetsov et al. 2018). NGS technologies allow a more accurate determination of each chromosome copy number and therefore, are much more sensitive than microarray-based approaches for

detecting segmental chromosomal abnormalities and mosaicism (Treff and Zimmerman 2017; Harton, Cinnioglu, and Fiorentino 2017; Fiorentino et al. 2014; Munne and Wells 2017). NGS limit of detection for mosaicism is approximately 20% (Maxwell and Grifo 2018), while aCGH resolution varies between 40 and 60%. The sensitivity of SNP array, qPCR and low-resolution NGS is very low (Mamas et al. 2012; Capalbo, Wright, et al. 2013). Besides its higher resolution and sensitivity, NGS is a cost-effective solution for PGT-A because a large number of samples (i.e., up to 96) can be simultaneously analysed, leading to a significant reduction of the testing costs compared with microarray-based techniques (Wells et al. 2014; Maxwell et al. 2016).

CONCLUSION

The published data demonstrated that SCM is an alternative approach to obtain embryo DNA. However, the huge heterogeneity in the study design and the frequent presence of extra-embryonic DNA in SCM weaken any conclusion on SCM-PGT diagnostic efficiency and clinical efficacy. Before considering its clinical use, studies using optimized procedures and larger sample sizes in different IVF centres should thoroughly investigate SCM-PGT reliability. Moreover, future studies should focus on the identification of cf-DNA origin to determine whether cf-DNA released in SCM is representative of the growing embryo. Moreover, the clinical outcomes (i.e., on-going pregnancy rate, miscarriage rate, and live birth) after SCM-PGT should be monitored before considering its implementation in the clinical routine.

LEGENDS

Figure 1. Invasive and non-invasive PGT. In invasive PGT, DNA could be extracted from embryonic cells obtained by cleaved embryo or blastocyst biopsies (A) or from cf-DNA released from the blastocoel fluid by blastocentesis or blastocoel collapse (B). In non-invasive PGT, embryonic DNA is obtained from cf-DNA released from the embryo in the spent culture medium. The embryo genetic status can be determined by quantitative Polymerase Chain Reaction (qPCR), Next-Generation Sequencing (NGS), Comparative Genomic Hybridization arrays (aCGH), or Single-Nucleotide-Polymorphism (SNP) analysis.

Figure 2: Flow chart summarizing the search strategy and study inclusion.

Table 1: Clinical and biological characteristics of the SCM samples in the selected studies. IVF: *in vitro* fertilization; ICSI: intracytoplasmic sperm injection; D: day(s); Z: zygote; CE: cleaved embryo; B: blastocyst; MCS: morphokinetic scores; PGS: preimplantation genetic screening; PGT: preimplantation genetic testing; NA: not available; SCM: spent culture medium.

Table 2: Detection and quantification of cf-DNA in SCM. SCM: spent culture medium, BF: blastocoel fluid, BC: blastocoel collapse, AH: assisted hatching, D: day(s), nDNA: nuclear DNA, mtDNA: mitochondrial DNA, PBB: polar body biopsy, TE: trophectoderm, ns: not significant, IQR: interquartile range, NA: not available.

Table 3: Concordance rates between SCM-PGT and iPGT. BF: blastocoel fluid, BC: blastocoel collapse, AH: assisted hatching, PB: polar body, TE: trophectoderm, D: day(s), nDNA: nuclear DNA, mtDNA: mitochondrial DNA, WE: whole embryo, SEA: Southeast Asia deletion, NA: not available.

Figure 3: SCM-PGT benefits/concerns and main limitations of the selected studies.

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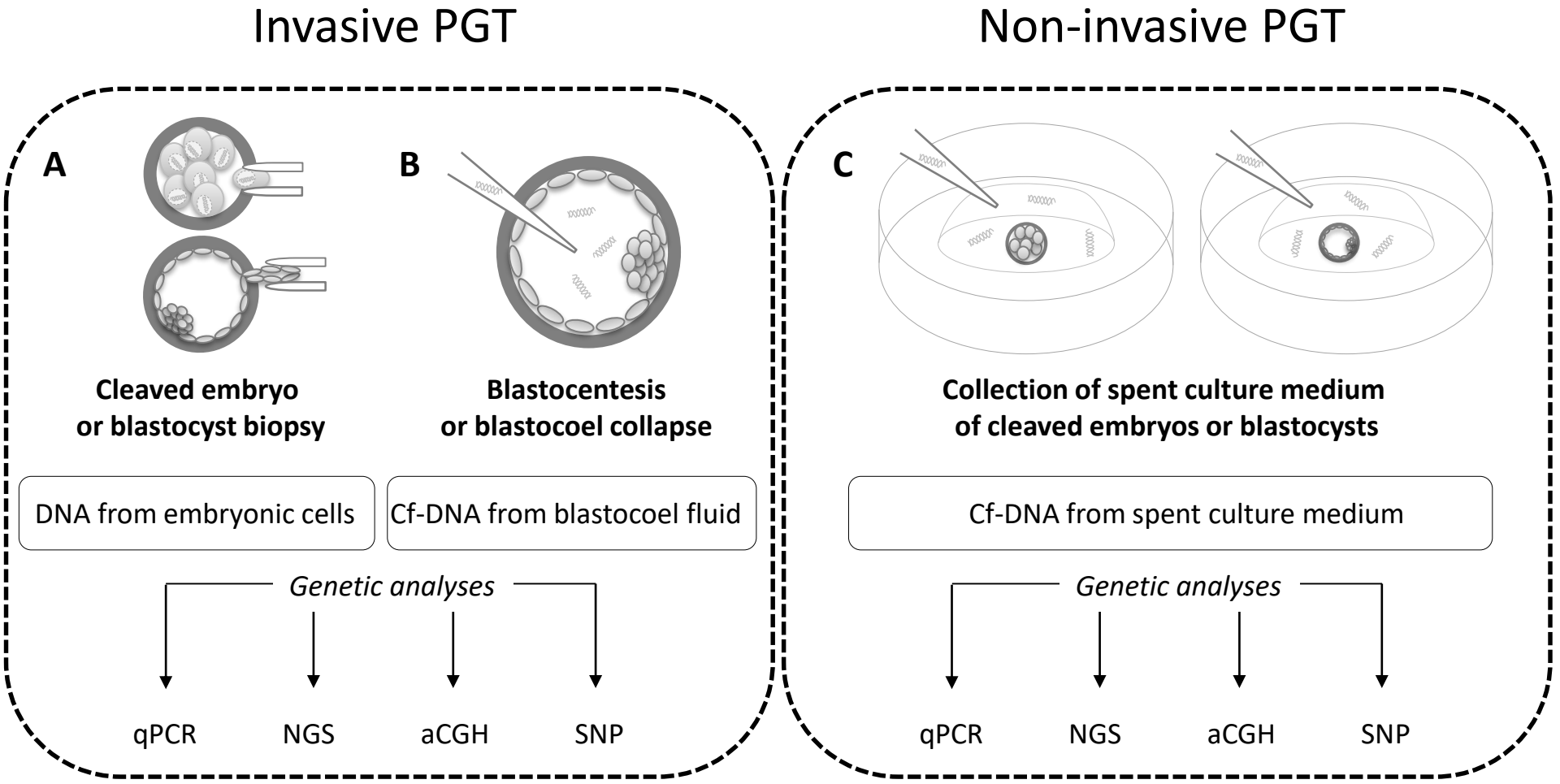
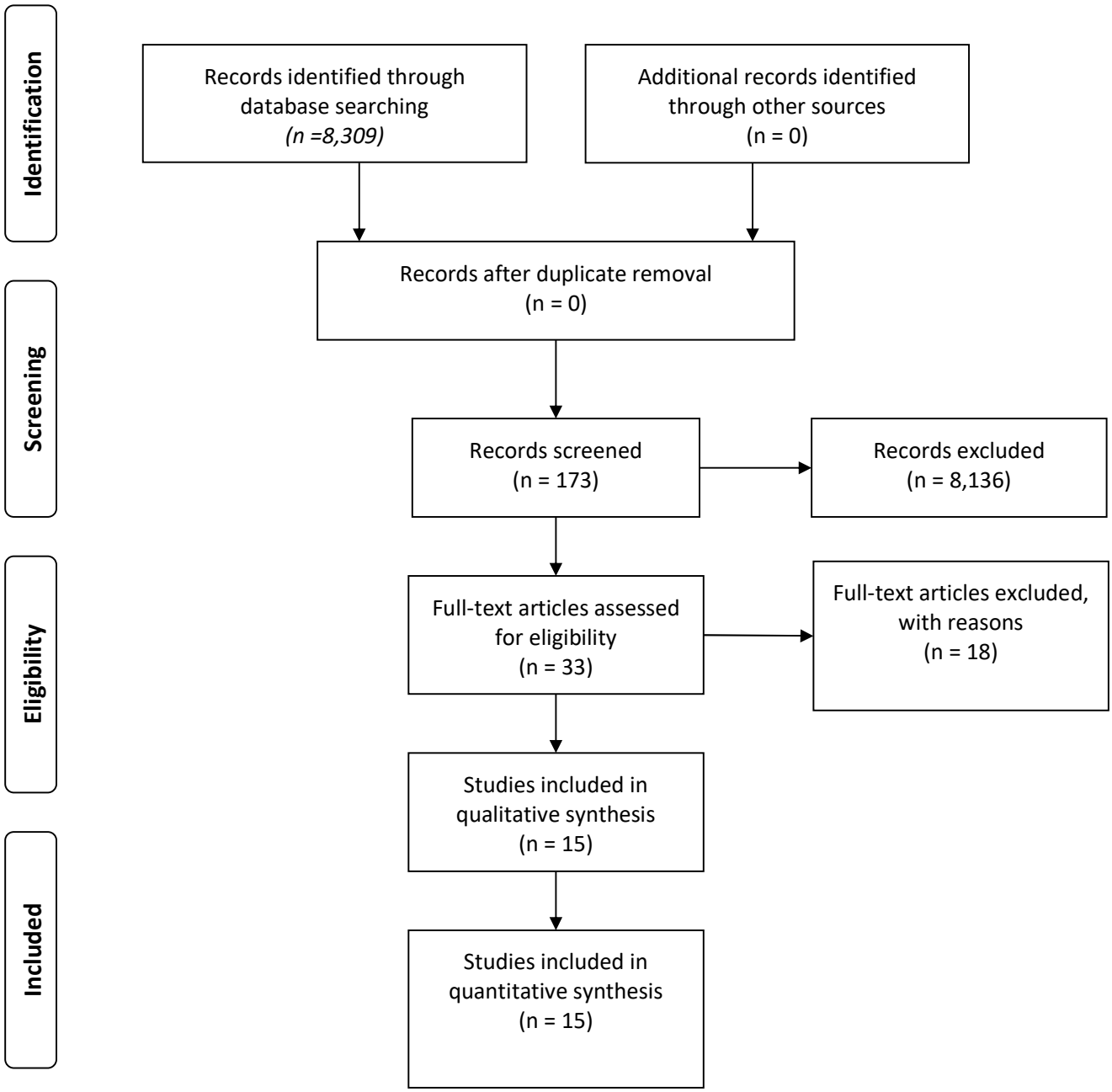

Invasive PGT
Non-invasive PGT
A
B
C
Cleaved embryo
or blastocyst biopsy
Blastocentesis
or blastocoel collapse
Collection of spent culture medium
of cleaved embryos or blastocysts
DNA from embryonic cells
Cf-DNA from blastocoel fluid
Cf-DNA from spent culture medium
Genetic analyses
Genetic analyses
qPCR
NGS
aCGH
SNP
qPCR
NGS
aCGH
SNP

Figure 1



From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit www.prisma-statement.org.

Figure 3

SCM-PGT benefits

- Non-invasiveness
- Easy, safe and quick procedure
- All embryos could be assessed

SCM-PGT concerns

- Low cf-DNA concentration
- Control of DNA contamination
- Origin of cf-DNA?

Limitations of the included studies

- Low sample size
- Mixed origin of embryos
- Partial collection of the SCM volume
- Absence of adequate negative controls
- SCM contamination by exogenous and/or maternal DNA
- Polar body/Trophectoderm biopsy as DNA control

TABLE 1

Study	Couples	IVF/ICSI	Nature of samples	Embryo characteristics			Release of cf-DNA	
				Fresh or frozen/thawed	Embryo quality	Embryo manipulation before SCM collection	Embryo stage (days post-fertilization)	Incubation time (days)
Stigliani, 2013	-	ICSI	SCM	Fresh	Top, fair and poor MCS	-	CE (D1 to D3)	1 to 2
Assou et al., 2014	-	-	SCM	-	-	-	CE and B (D3/5/6)	-
Galluzzi et al, 2015	-	-	SCM	Fresh	-	-	Z, CE, B (D0 to D6)	2 to 3
Wu et al., 2015	41	ICSI	SCM	Fresh	Top, fair and poor MCS	+/- blastomere biopsy	CE and B (D3 to D6)	1
Xu et al., 2016	17	ICSI	SCM	Fresh or frozen	Top and fair MCS	-	CE and B (D3 to D5)	2
Shamonki et al., 2016	7	ICSI	SCM	Fresh	Top and fair MCS	Assisted hatching	CE and B (D3 to D5)	2 to 3
Liu et al., 2017	7	ICSI	SCM	Fresh	Top, fair and poor MCS	no	B (D? to D5)	5 ?
Hammond et al., 2017	70	ICSI	SCM and SCM+BF	Fresh	Top and fair MCS	+/- blastocoel collapse	CE and B (D3 to D6)	2 to 5
Yang et al., 2017	-	ICSI	SCM and SCM+BF	Fresh	-	+/- blastocoel collapse	Z, CE and B (D0 to D6)	1 to 3
Feichtinger et al., 2017	-	ICSI	SCM	Fresh	Top, fair and poor MCS	Assisted hatching and polar body biopsy	B (D5)	5
Vera-Rodriguez et al., 2018	42	ICSI	SCM	Fresh	Top and fair MCS	Assisted hatching	CE and B (D3 to D5)	2
Kuznyetsov et al., 2018	35	ICSI	SCM+BF	Fresh or frozen	-	Trophectoderm biopsy, blastocoel collapse (simple or double)	CE and B (D4 to D7)	1 to 2
Li et al., 2018	-	ICSI	SCM+BF	Fresh	Spare embryos with good MCS	Assisted hatching and blastocoel collapse	CE and B (D3 to D5)	2
Ho et al., 2018	9	ICSI	SCM	Fresh or frozen	Top, fair and poor MCS	+/- Assisted hatching	Z, CE and B (D1 to D5)	2 to 4
Capalbo et al., 2018	26	ICSI	SCM	Fresh	-	no	B (D1 or D3-D5)	2 to 5

TABLE

Study	Samples		Embryo manipulation before sample collection	Embryo secretion of cf-DNA		mtDNA/nDNA	Amplification technology	Amplification it	enetic DNA analysis	DNA quantification method	DNA detection	DNA quantification concentration or le el	p alue	uality of studies (mN S)
	Nature	n		Timing of secretion	Incubation time									
Stigliani, 2013	SCM	326	-	D1-D2 + D1-D3	1-2 days	nD A	CR-based	Genome le	CR	Single copy region of non-transcribed D A/R Ase	62.9 (205/326)	41 pg to 1.8 ng	-	7/12
		322				mtD A	MDA	Repli-G	CR	*NDI* (detection) and *MT-7S* D-loop region (uantification)	98.8 (322/326)	mtD A/gD A copy number ratio 5 to 5221 (average 394)	-	
Assou et al., 2014	SCM	3	-	D3/5/6	ns	nD A	no	-	CR	*TSPY1* (chr. Y)	100 (3/3)	-	-	2/12
Wu et al., 2015	SCM	61	-	D3-D4	1 day	nD A	no	-	CR	*HBA (SEA mutation)*	19.67 (12/61)	14.24 4.76 pg/ L	-	9/12
		61		D4-D5	1 day						90.16 (55/61)	48.78 20.45 pg/ L	ns	
		26		D5-D6	1 day						88.46 (23/26)	54.35 22.78 pg/ L		
		202	Blastomere biopsy	D5-D6	1 day						88.6 (179/202)	-	-	
Galluzzi et al, 2015	SCM	32	no	D0-D3	3 days	nD A	no	-	CR	*TBC1D3 (chr. 17)*	93.7 (30/32)	80 70 pg	p 0.05	5/12
		54		D3-D5/6	2-3 days						94.4 (51/54)	99 113 pg		
		32		D0-D3	3 days					*TSPY1* (chr. Y)	6.7 (2/32)	-	-	
		54		D3-D5/6	2-3 days						41.2 (21/54)	-	-	
		8	-	D3-D5/6	2-3 days	nD A	MALBAC-like	ico LEX	CR	*MTHFR* (chr.1)	62.5 (5/8)	-	-	
Xu et al., 2016	SCM	42	Vitrification	D3-D5	2 days	nD A	MALBAC	MALBAC	GS	A	100 (42/42)	-	-	8/12
		8	no								100 (12/12)	-	-	
Shamonki et al., 2016	SCM	57	AH	D3-D5/6	2-3 days	nD A	MDA	Repli-G	aCGH	ubit (broad range D A kit)	96.49 (55/57)	2 642 ng/ L (mean 66 ng/ l)	-	11/12
		6									100 (6/6)	52 642 ng/ L	-	
Liu et al., 2017	SCM	88	no	D?-D5	5 days?	nD A	MALBAC	MALBAC	GS	Agilent 2100 Bioanalyser and gel electrophoresis	90.90 (80/88)	10.6-78.2 ng/ l (mean 26.15ng/ l)	-	9/12
		70									90.00 (63/70)	mean 60.15ng/ l	-	
		10									80 (8/10)	-	-	
Hammond et al., 2017	SCM	102	no	D1-D3	2 days	nD A	no	-	Digital CR	*RNAse P* (chr. 14)	-	2 copies	**p**	10/12
				D3-D5	2 days						-	3 copies		
		52		D1-D6	5 days						-	6 copies	**p 1**	
	SCM	102	no	D1-D3	2 days	mtD A				*MT-ND1* (mtD A)	-	90 +/- 22 copies		
				D3-D5	2 days						-	259 +/- 42 copies	**p 1**	
		52		D1-D6	5 days						-	625 +/- 118 copies	**p 1 s D1-D** , ns vs D3-D5	
Yang et al., 2017	SCM	5	no	D0-D1	1 day	nD A	no	-	CR	*SRY* (chr. Y)	?	-	-	5/12
		4		D0-D2	2 days						50 (2/4)	-	-	
		9		D0-D3	3 days						100 (9/9)	-	-	
		4		D3-D4	1 day						100 (4/4)	-	-	
		4		D3-D5	2 days						100 (4/4)	-	-	
		4		D3-D6	3 days						100 (4/4)	-	-	
	SCM+BF	3	BC	D3-D6	3 days						100 (3/3)	-	-	
Feichtinger et al., 2017	SCM	22	AH and BB	D0-D5	5 days	nD A	MALBAC-like	Sure LEX	aCGH	ubit and gel electrophoresis	81.8 (18/22)	21.33 ng/ l (range 6.7 41)	-	8/12
Vera-Rodriguez et al., 2018	SCM	53	AH	D3-D5	2 days	nD A	no	-	CR	resD ASE Human Residual D A uantitation Kit	-	6.7 pg l R 3.2 12.6pg	-	9/12
		60					MALBAC-like	Sure LEX	CR	resD ASE Human Residual D A uantitation Kit	-	4.9 g (l R 3.4 6.2 g)	-	
		56							GS	Agilent 2100 Bioanalyzer and ubit	91.1 (51/56)	-	-	
Kuznyetsov et al., 2018	SCM+BF	28	Vitrification, +/- TE biopsy (24/28), double BC	D5/6 + 24h post thawing	1 day	nD A	MALBAC-like	Sure LEX	GS	ubit	100 (28/28)	21.7 5.2 ng/ l (range 10.5 to 44.0 ng/ l)	-	12/12
		19	no	D4-D5/6	1/2 days						100 (19/19)	15.2 2.7 ng/ l (range 6.3 to 36.0 ng/ l)	-	
Li et al., 2018	SCM+BF	39	AH and BC	D3-D5	2 days	nD A	MALBAC	MALBAC	GS	anodrop	97.5 (39/40)	58.03 35.87 ng/ l	-	10/12
Ho et al., 2018	SCM	41	Slow freezing, +/- AH	D1-D3	2 days	nD A	MALBAC-like	ico LEX	GS	ubit	97.6 (40/41)	111 ng/ L (l R 96.6 120 ng/ L)	**p 1**	9/12
				D1-D5	4 days						97.6 (40/41)	105 ng/ L (l R 41.4 117 ng/ L)		
		20	+/- AH	D0-D5	5 days						100 (20/20)	88.7 ng/ L (l R 84.3 92.7 ng/ L)	-	

TABLE

Study	mtDNA/nDNA	Samples (n)	Culture	Incubation time	Embryo manipulation before sample	Embryo stages	enetic DNA analysis	Sequencing technology	Analysis system	enome position	Interpretable results of amplified DNA from SCM	Embryo DNA reference	eneral concordance rate ( )	Full concordance rate ( )	p alue	Study quality (mN S score)
Galluzzi et al, 2015	nD A	2	D0-D3	3 days	no	-	- CR	-	-	*TSPY1* (chr. Y)	-	2 TE biopsies (3-5 cells)	50 (1/2)	50 (1/2)	-	6/12
		2	D3-D5/6	2-3 days	no	-					-		100 (2/2)	100 (2/2)	-	
		5	D3-D5/6	2-3 days	no	-					-	5 corresponding pregnancies	100 (5/5)	100 (5/5)	-	
		8	D3-D5/6	2-3 days	no	-				*MTHFR* (chr.1)	100 (2/2)	2 TE biopsies (3-5 cells)	50 (1/2)	50 (1/2)	-	
Xu et al., 2016	nD A	42	D3-D5	2 days	Vitrification	Blastocysts	GS	synthesis	HiSe 2500 (Illumina)	24-chromosome screening	100 (42/42)	42 WE	85.7 (36/42)	57.1 (24/42)	-	12/12
		8			no	-					100 (8/8)	8 live births	100 (8/8)	-	-	
		3			no	Blastocysts					100 (3/3)	2 WE and 1 live birth	100 (3/3)	100 (3/3)	-	
		5			no	Blastocysts					100 (5/5)	5 live births	100 (5/5)	-	-	
Shamonki et al., 2016	nD A	6	D3-D5/6	2-3 days	AH	Blastocysts	aCGH	-	A (Agilent)	24-chromosome screening	33.3 (2/6)	2 TE biopsies	100 (2/2)	100 (2/2)	-	10/12
Liu et al., 2017	nD A	31	D7-D5	5 days?	no	Blastocysts	GS	synthesis	HiSe 2500 (Illumina)	24-chromosome screening	- -	31 TE biopsies (3-6 cells)	83.87 (26/31)	64.6 (20/31)	-	8/12
		25				Arrested embryos					-	25 embryo biopsies	76 (19/25)	44.00 (11/25)	-	
		8				Blastocysts and arrested embryos				*HBB* (IVSII654 mutation) (chr. 11)	100 (8/8)	8 TE biopsies (3-6 cells)	87.5 (7/8)	87.5 (7/8)	-	
Feichtinger et al., 2017	nD A	18	D0-D5	5 days	AH and BB	Mi of arrested embryos and blastocysts	aCGH	-	24 sure (Illumina)	24-chromosome screening	100 (18/18)	18 B biopsies	72.2 (13/18)	22.2 (4/18)	-	8/12
Vera-Rodriguez et al., 2018	nD A	56	D3-D5	2 days	AH	Blastocysts	GS	ion detection	Ion GM (Life Technologies)	24-chromosome screening	100 (51/51)	51 TE biopsies (4-6 cells)	54.9 (28/51)	5.9 (3/51)		11/12
Kuznyetsov et al., 2018	nD A	24	D5/6 + 24h post-thawing	1 day	Vitrification, +/- TE biopsy (24/28), double BC	Blastocysts	GS	synthesis	MiSe (Illumina)	24-chromosome screening	100 (24/24)	24 TE biopsies (4-6 cells)	87.5 (21/24)	66.7 (16/24)		12/12
		28				Blastocysts					100 (28/28)	28 WE	96.4 (27/28)	78.5 (22/28)		
		19	D4-D5/6	1/2 days	no	Blastocysts					100 (19/19)	19 TE biopsies (4-6 cells)	100 (19/19)	73.7 (14/19)		
Li et al., 2018	nD A	39	D3-D5	2 days	AH and BC	Spare embryos	GS	synthesis	HiSe 2500 (Illumina)	24-chromosome screening	100 (39/39)	38 TE biopsies (3-5 cells)	76.3 (29/38)	36.8 (14/38)		10/12
		39									100 (39/39)	38 WE	78.9 (30/38)	39.5 (15/38)		
Ho et al., 2018	nD A	16	D1-D3	2 days	Freezing/thawing (n 16), AH (n 8)	Developing and arrested embryos	GS	ion detection	Ion S5 Se uencer (Life Technologies)	24-chromosome screening	40.0 (16/40)	16 WE	56.3 (9/16)	-		8/12
		33	D1-D5	4 days	Freezing/thawing (n 33), AH (n 16)	Developing and arrested embryos					82.5 (33/40)	33 WE	45.5 (15/33)	-		
		13 out of 33				Arrested embryos					-	13 WE	18.2 (2/13)	-	p	
		20 out of 33				Blastocysts					-	20 WE	65.0 (13/20)	-		
		40	D0-D/D1-D5	4/5 days	Mi of fresh (n 20) and freezed/thawed embryos (n 20). AH in 28/40 embryos	Blastocysts					100 (40/40)	26 TE	65 (26/40)	-		
Capalbo et al., 2018	nD A	72	D1-D5 or D3-D5)	2 or 4 days	no	Blastocysts	- CR	-	-	11 monogenic mutations and S	89.7 (339/378 loci)	72 TE biopsies (3 10 cells)	-	66.4 (225/339 loci)	-	11/12