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Review Article

PGT-A for embryos for transfer and to improve clinical outcomes in terms of embryo implantation, and recommendations embryo biopsy for at blastocyst stage

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ABSTRACT

The development of Preimplantation Genetic Testing (PGT) is evolving fast, and best practice advice is essential for regulation and the development of new papers outlining recommendations for good practice in PGT was necessary.

Background: Preimplantation genetic testing (PGT) is widely used today in In-vitro Fertilization (IVF) centers over the world for selecting euploid embryos for transfer and to improve clinical outcomes in terms of embryo implantation, clinical pregnancy, and live birth rates. Methods: We report the current knowledge concerning these procedures and the results from different clinical indications in which PGT is commonly applied. Biopsies for PGT. Finally, genetic and clinical significance of embryo mosaicism are illustrated. Preimplantation Genetic Testing for Aneuploidies (PGT-A) may be limited to women of advanced maternal age or with recurrent pregnancy loss, the fluid taken at the time of embryo biopsy can be analysed for any frozen embryo as well as PGT-A embryos. The current paper provides recommendations on the technical aspects of embryo biopsy and covers recommendations on the biopsy procedure, cryopreservation and laboratory issues and training, in addition to technical aspects and strengths and limitations specific for currently used techniques at different stages (polar body, cleavage stage and blastocyst biopsy).

Keywords: Preimplantation genetic screening • IVF • Cryopreservation of biopsied oocytes biopsy laboratory infrastructure • Mosaicism • Trophoectoderm biopsy • Mosaic blastocysts transfer

Abbreviations: PGT: Preimplantation Genetic Testing; IVF: In-vitro Fertilization; SET: Single Embryo Transfer; PGT-A: Preimplantation Gentic Testing for Aneuploidies; FISH: Fluorescence In-situ Hybridization; ACGH: Array-Comparative Genomic Hybridization; NGS: Next Generation Sequencing; RTQ-PCR: Real Time Quantitative Polymerase Chain Reaction; ICSI: Intracytoplasmic Sperm Injection; TE: Trophoectoderm; BF: Blastocyst Fluid; WGA: Whole Genome Amplification; AMH: Anti-Mullerian Hormone; AMA: Advanced Maternal Age; FTET: Frozen Thawed Embryo Transfer; RPL: Recurrent Pregnancy Loss; PGT-M: Preimplantation Genetic Testing for Monogenic Disease; DOR: Diminished Ovarian Reserve; RIF: Repeated Implantation Failure; SMF: Severe Male Factor; KS: Klinefelter Syndrome; WHO: World Health Organization; NOA: Non-obstructive Azoospermia; DFI: DNA Fragmentation Index; SCOS: Sertoli Cell-Only Syndrome

Introduction

IVF is a reproductive technique whose success rate depends on several steps: ovarian stimulation, egg retrieval, embryo culture, and transfer. Embryo implantation is one of the most critical point in every IVF program and transfer of a vital embryo in a receptive endometrium is essential for achieving a pregnancy in an assisted reproduction cycle. Despite many improvements reached today the process of embryo implantation is still very ineffective [1]. Therefore, the selection of the best embryo to transfer is the main challenge to face, mostly when

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a Single Embryo Transfer (SET) program is adopted for different clinical reasons. As currently practiced, the embryo that is chosen for transfer is selected on morphologic grading criteria, which has significant inter- and intraobserver variability [2]. At the cleavage stage, the number of cells, their symmetry, and the presence of cellular fragments are evaluated. At the blastocyst stage, the evaluated parameters are blastocyst expansion and the inner cell mass and trophoectoderm appearance. Today, there is a wide consensus that the microscopic appearance of an embryo is weakly correlated with its viability [3,4]. Thus, a variety of non-invasive methods, such as time-lapse imaging for embryo morphokinetics [5], proteomic [6], and metabolomic [7] study, was proposed to assess the embryo quality. Extending embryo culture to the blastocyst stage was shown to improve outcomes from SET [8], although morphologically normal blastocysts still retain a significant risk of aneuploidy [9–12]. Therefore, the clinical outcomes from SET have been demonstrated to be lower in several randomized controlled trials performed to date and confirmed by subsequent meta-analysis [13,14]. The transfer of multiple embryos is frequently the previous terms of Preimplantation Genetic Diagnosis (PGD) and Preimplantation Genetic Screening (PGS) have been replaced by the term Preimplantation Genetic Testing (PGT), following a revision of terminology used in infertility care. PGT is defined as a test performed to analyse the DNA from oocytes (polar bodies) or embryos (cleavage stage or blastocyst) for HLA typing or for determining genetic abnormalities. This includes PGT for aneuploidy (PGT-A), PGT for monogenic/single gene defects (PGTM) and PGT for Chromosomal Structural Rearrangements (PGT-SR). PGT for chromosomal numerical aberrations of high genetic risk are included within PGT-SR in the data collections of the ESHRE PGT consortium. PGT began as an experimental procedure in the 1990s with Polymerase Chain Reaction (PCR)-based methods used for sex selection and the detection of monogenic diseases. Interphase Fluorescence In-situ Hybridisation (FISH) was introduced a few years later and became the standard method for sexing embryos and for detecting numerical and structural chromosomal aberrations. Genome-wide technologies began to replace the gold standard methods of FISH and PCR over the last decade and this trend was most apparent for PGT-A. PGTA has been carried out mainly for In-vitro Fertilization (IVF) patients with original aims of increasing pregnancy rates per embryo transfer and decreasing miscarriage rates. Other outcome measures such as increasing elective single embryo transfer and reduced time to pregnancy have been added more recently. Cited indications for PGT-A include Advanced Maternal Age (AMA), Recurrent Implantation Failure (RIF) and Severe Male Factor (SMF) and couples with normal karyotypes who have experienced Recurrent Miscarriage (RM). The value of the procedure for all IVF patients and/or appropriate patient selection remains an ongoing discussion, but this is outside the scope of this manuscript. The goal of this series of papers is to bring forward best practices to be followed in all types of PGT services, offering PGT-A as well as PGT-M and PGT-SR. In order to take PGT to the same high-quality level as routine genetic testing, guidelines for best practice have been designed by several societies. The PGD International Society has drafted guidelines (The Preimplantation Genetic Diagnosis International Society) while the American Society for Reproductive Medicine reviewed PGT practice in the USA Practice Committee of the Society for Assisted Reproductive Technology and Practice Committee of the American Society for Reproductive Medicine (2008) and published several opinion papers (on blastocyst culture, embryo transfer and on PGT-A). The first guidelines of the ESHRE PGT Consortium were published in 2005, as one of the missions of the Consortium was to bring overall standardisation and improve quality standards. In collaboration with the Cytogenetics European Quality Assessment (CEQA) and the UK National External Quality Assessment Service (UKNEQAS), now together in Genomics Quality Assessment (GenQA), the ESHRE PGT Consortium also initiated External Quality Assessment (EQA) schemes to provide an independent evaluation of laboratories and help them improving their techniques and reports. A review of the original guidelines yielded four sets of recommendations on different aspects of PGT: One on the organisation of PGT and three relating to the methods used: embryo biopsy, amplification based testing and FISH-based testing. These four guidelines are now being updated and extended, taking into account the fast changes in the provision of PGT services. In these updated guidelines, the laboratory performing the diagnosis will be referred to as the PGT centre and the centre performing the IVF as the IVF centre. General aspects of PGT, including patient selection, counselling, pregnancy and children follow-up and transport PGT, will be covered in the paper on organisation of PGT. Technical recommendations for embryo biopsy and tubing will be covered in the paper on embryo biopsy. Recommendations for genetic testing will be covered in the papers on detection of numerical and structural chromosomal aberrations, and on detection of monogenic disorders. utilized in clinical practice to improve the chance of implantation, but this approach increases the risk of multiple pregnancies [15,16]. At the same time, several studies have demonstrated that embryo aneuploidy is the most important reason of IVF failure, enhancing the importance of Preimplantation Genetic Testing for Aneuploidies (PGT-A) as a method for selecting chromosomally healthy embryos [17-19]. Aneuploidies in human embryos are strictly correlated with female age [20] and are derived from chromosomal errors that can occur at different levels. Meiotic errors occur during oogenesis: the prolonged arrest of oocyte development in prophase results in a degradation of the meiotic apparatus. Mitotic errors happen after fertilization, usually during the first mitotic divisions and lead to embryo mosaicism. Sperm aneuploidies, generally correlated with sperm quality and DNA fragmentation, are less common if compared to oocytes ones, but their incidence in embryo aneuploidy is reported to be high [21]. PGT-A was introduced for the first time in the 1993 to select euploid embryos to transfer and improve assisted reproductive results [22]. However, the first generation PGT was demonstrated to be less effective in improving IVF Live Birth (LB) rates and reducing miscarriage rates [23] mainly due to the incomplete assessment of chromosomal status and undiagnosed mosaicism deriving from post-zygotic cleavage division errors in day-3 embryo [24]. In fact, in the beginning this screening was performed using Fluorescence In-situ Hybridization (FISH), which analyzed only a reduced number of chromosomes. The need to investigate embryos ploidy status led to the development of different

techniques for the analysis of the whole chromosomal panel, such as Array-Comparative Genomic Hybridization (aCGH), Next Generation Sequencing (NGS), and Real Time Quantitative Polymerase Chain Reaction (rtq-PCR). The biopsies for the analysis can be removed from the oocyte, collecting the first and/or second polar body or from the cleavage stage embryo, removing some blastomeres or from the blastocyst, collecting some trophoectoderm cells. These techniques can be applied for different indications in which the transfer of euploid embryo might improve the clinical outcomes.

Laboratory issues related to biopsy

Prior to the biopsy procedure, work surfaces, equipment and materials should be cleaned and decontaminated with disinfectants with proven compatibility and efficacy for use in an IVF laboratory. During PGT-related procedures, protective clothing should be worn, including full surgical gown (clean, not sterile and changed regularly), hair cover/hat, face mask (covering nose and mouth) and preferably shoe covers or dedicated shoes. Gloves should be worn at all times and changed frequently. Gloves should be powder-free and well-fitting (e.g. nitrile, but not vinyl). Insemination and culture, Intracytoplasmic Sperm Injection (ICSI) is preferable for PGT, to minimise the risk of both maternal contamination from residual cumulus cells and paternal contamination from surplus sperm attached to the ZP. Careful removal of cumulus cells (denudation) and rinsing of oocytes prior to ICSI and of zygotes in case of IVF after fertilisation check, are critical to avoid residual maternal contamination in the biopsy samples. Until time of biopsy, routine IVF culture conditions apply. The most adequate culture conditions, strategies and media should be used. If available, time-lapse imaging systems with a 'closed' culture system may be adopted to limit the exposure of the embryos to sub-optimal conditions and more easily decide on the optimal time for biopsy. Following biopsy, oocytes and embryos should be thoroughly rinsed to remove the biopsy medium before culture or cryopreservation. To culture embryos individually, the use of multiple-well dishes or droplets in separate dishes is advisable, to prevent mixing of embryos due to accidental movement during handling.

Biopsy laboratory infrastructure, equipment and materials

The embryology laboratory design should include a dedicated area for biopsy. A separate biopsy laboratory room may be advisable to provide adequate functionalities in IVF centres with high workload. The biopsy laboratory, whether it is a dedicated area or a room, should be designed taking into account all safety and environmental standards (air quality, positive pressure, laboratory access etc) as recommended in the 'Revised Guidelines for good practice in IVF laboratories', section 3 called 'Laboratory safety' to ensure good laboratory practice and to minimise any damaging effects on biological material. It is advised that tubing is performed in a dedicated area or room, in close proximity to the biopsy area (see section 'Sample collection'). Equipment The biopsy equipment set-up includes an inverted microscope with heated stage and three-dimensional micromanipulators and microinjectors (air or oil), placed on antivibration pads, equivalent to a setup for ICSI procedures. In addition, a stereoscope (for transferring oocytes/embryos in biopsy dishes and for tubing) and incubators should be available adjacent to the working area. A CE mark is recommended for all equipment, taking into consideration local legislation. Special equipment such as a laser might be required for assisted hatching and blastocyst biopsy. The laser is usually included in a $\times 25$ or $\times 40$ objective of an inverted microscope and piloted by a software and camera. The laser can be controlled either using mouse or foot switch.

Cryopreservation of biopsied oocytes/embryos

There are several situations when oocytes/embryos may be frozen in cases of PGT, depending on laboratory strategy and local regulations: (i) Prior to the biopsy (e.g. accumulation of oocytes/embryos; surplus oocytes/embryos from previous non-PGT cycles); (ii) After the biopsy (i.e. testing platforms often require cryopreservation as a mandatory step to give time for the genetic laboratory to analyse the samples); (iii) Or after the biopsy and diagnosis (e.g. fresh embryos have been transferred but supernumerary tested embryos need to be stored). At any stage along preimplantation development, cryopreservation via vitrification is recommended and the same protocol applies to biopsied and non-biopsied embryos. Biopsied embryos must be vitrified individually in a cryo-support properly labelled, and witnessing is mandatory. Multiple vitrification-warming cycles may be necessary in a minority of PGT cases; however, the influence of this approach on embryo viability/implantation and clinical outcomes still needs further investigation. It is recommended that each centre decides its own policy regarding the cryopreservation/vitrification of PGT embryos, based on its experience and performance.

Moreover, proteins, mitochondrial DNA, and miRNAs have also been detected in the blastocoel fluid. Prior literature indicates that the origin of these molecules may potentially be remnants of cells from the developing blastocyst that underwent apoptosis during.

In accordance, microRNAs, some of which were linked to apoptosis, and extracellular vesicles were also found in blastocoel fluid from human preimplantation embryos. The discovery of microRNA linked to apoptosis as well as extracellular vesicles only further provide support to the existence of a preimplantation embryo self-correction mechanism. Moreover, the link to apoptosis presents a probable mechanism which potentially purges developing preimplantation embryos of aneuploid cells. Recent literature points to an increased interest in using the information provided by blastocoel cfDNA alongside PGT-A as a cumulative measure of preimplantation embryo quality. Several reports have postulated that competent preimplantation embryos may be identified via cfDNA content in the blastocoel fluid or spent media. This interest has recently led to increased research which analyzes the overall potential of specific cfDNA studies to reveal specific embryo insights. While some studies have reported at least a limited concordance between the chromosomal status detected using blastocoel cfDNA in comparison to PGTA from embryonic TE biopsy, there is not enough literature or evidence to ensure that the blastocoel cfDNA analysis accurately confirms ploidy status. The advantages of an analysis utilizing blastocoel cfDNA data rather than PGT-A are obvious (primarily the ability to test for aneuploidy without performing embryo biopsy), but the theoretical concept remains unproven, as the pragmatic concordance has yet to reach a satisfactory level. A truly noninvasive approach to assess preimplantation embryo ploidy status (non-invasive

PGT-A) would involve analysis of spent media from preimplantation embryos cultured from early cell cleavage stages to the blastocyst stage that did not undergo embryo biopsy. A proof-of-concept study thawed and cultured previously frozen donated blastocysts (with known ploidy status from embryo biopsy) and then collected their spent media, which likely contained cfDNA from blastocoel fluid. The spent media was then assessed for PGT-A and revealed high concordance with the PGT-A results from the TE biopsy. Though this study suggests that non-invasive PGTA from spent media is promising, the manner in which the media was collected in the study is not a routine procedure for IVF cases. However, combining PGT-A from TE biopsy with embryonic cfDNA analysis (DNA obtained from spent blastocyst medium) has improved implantation rates and additional analyses on blastocoel components may further enhance implantation rates.

The single step procedure would seem to be more convenient, since pronuclei detection allows for analyzing only fertilized oocytes, reducing costs and time wasting. Furthermore, combining the first and second PB biopsy could result in an improved abnormalities detection rate [25]. However, the PB biopsy only provides maternal genetic information and does not consider parental or mitotic division abnormalities [26]. European Society of Human Reproduction and Embryology started in 2012 a multicenter randomized clinical trial to establish the effectiveness of PGT-A performed with PB biopsy. The aim of the study was to evaluate whether the analysis of 23 chromosomes in the first and second polar body, and the selection of euploid embryos for transfer, increased live birth rate within one year, in women in advanced maternal age as compared to cycles without PGT-A. From June 2012 to December 2016, 205 women were assigned to cycles with PGT-A, and 191 to cycles without PGT-A (control group). However, the LB rate was not different among the two groups: 50 out of 205 (24%) in the PGT-A group and 45 out of 191 (24%) in the control group. PB biopsy has the benefit of providing a long time to perform genetic testing without requiring embryo cryopreservation despite being time-consuming [25-27] and less cost-effective per LB rate [28] and, therefore, it is the only genetic testing strategy available in many countries with legal restriction on embryo genetic assessment and cryopreservation

Blastomere biopsy

Blastomere biopsy is usually performed when the embryo is made of about six or eight cells, which usually happens 72 h after insemination. The first step to perform the biopsy is to open the zona using tyrode acid, mechanical piercing, or laser-assisted hatching. Laser assisted zona drilling and the use of calcium-magnesium free media to weaken cell cohesion is the most widespread procedure according to the report of ESHRE PGT consortium in 2011 [29]. It is possible to remove one to two blastomeres. Two cells biopsy is more accurate, but it could affect embryo vitality, since it results in the removal of about 30% of the whole embryo. One cell biopsy, on the other side, could result in misleading or incorrect diagnosis [30]. Other studies [31,32] have suggested that removing of more blastomeres has negative effects on embryo development, which leads to reduced implantation rates, but it provides a higher diagnostic efficiency when compared with the removal of only one cell. However, this technique is compatible with fresh embryo transfer on day 5 or 6 of embryo development, given that genetic results will usually be available one to two days after blastomere biopsy (Figure 1).

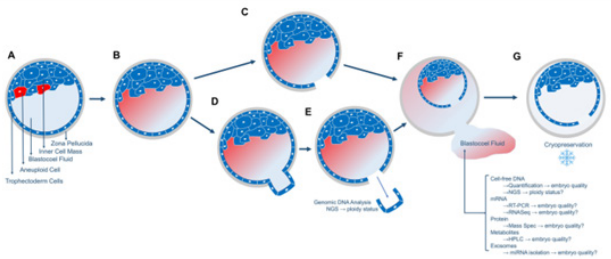

Figure 1: Overview of the collection of blastocoel fluid. (A) The day 5/6 blastocyst includes the fluid-filled blastocoel cavity in contact with the inner cell mass. Red cells in the inner cell mass represent aneuploidy cells that will or has undergo apoptosis. (B) The cells that underwent apoptosis released their contents (cell-free DNA, cryopreservation by using a laser to release the blastocoel fluid into the surrounding media drop. (D,E) Blastocysts that will undergo embryo biopsy for PGT-A will typically have 3-10 trophectoderm (TE) cells removed visa laser-assisted cell lysis and pipette suction, with the biopsied cells sent off-site for genetic analysis. (F) Following either micromanioulation process, the blastocoel fluid is expelled from the blastocyst into the surrounding media and this blastocoel conditioned media can be collected and stored for subsequent analyses of the molecules as listed. (G) The blastocyst is cryopreserved and stored. Blastocysts are thawed once PGT-A results are known for embryo transfer.

Trophoectoderm biopsy

The blastocyst is composed of two different cell types: the inner cell mass, which will evolve in fetal tissues, and the Trophoectoderm (TE) considered the precursor of future placenta. The advantages correlated to TE biopsy are mainly three: first of all, TE is not involved in fetus formation, as it will form extra-embryonic tissues. The second benefit is that blastocyst stage embryos have already activated their genome, allowing for a more accurate analysis. Finally, a sample of about five-eight cells is needed for the test, determining a loss of about 10% of all of the cells forming the blastocyst (about 100–150). When compared to the cell mass loss determined by the removal of two blastomeric, this procedure seems to be much less invasive [33]. Blastocyst biopsy also implicates other practical advantages: embryos vitrified at this stage show a higher survival rate if compared with cleavage stage embryos. Therefore, it allows for postponing the transfer and even adopts a single embryo transfer policy reducing multiple pregnancies [34]. Three main approaches can be followed for TE biopsy: the first consist in opening the zona pellucida at cleavage stage using a laser-assisted drilling and then waiting for the formation of expanded or herniating blastocysts on day 5 Cleavage-stage zona drilling is performed to obtain a faster biopsy on herni-

ating blastocysts and reduce the chance of sudden collapse. Although being widely adopted, this procedure presents two main limitations; it entails two sessions of embryo manipulation outside the incubator and there is the concrete risk of having the inner cell mass herniating outside the zona. The second approach to TE biopsy is to leave the embryo in culture until blastocyst full expansion and then open the zona immediately before the biopsy, with assisted laser hatching. This strategy requires a single intervention on the embryo and the zona can be opened in a region far from the inner cell mass, reducing its involvement in the biopsy process [35]. The last method takes advantages of both the previous approaches: it consists of opening the zona when the blastocyst is fully expanded and then waiting for the TE herniation. Figure 2 shows the blastocysts biopsy laser assisted steps.

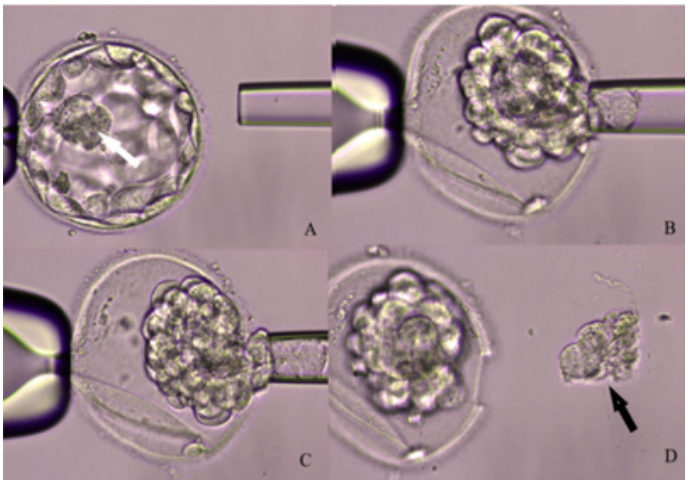

Figure 2: In the figure, the sequence of a blastocyst biopsy laser assisted is shown. The blastocyst is initially orientated by mean of the holding pipette in order to keep the inner cell mass as far as possible from the site of biopsy. (A) Subsequently, the biopsy pipette is introduced through a hole performed with laser in the zona pellucida and a little number of trophectoderm cells are gently aspirated. (B,C) Finally, the removed trophectoderm cells is transferred in a tube for the genetic analysis. (D) The white narrow indicates the inner cell mass. The black narrow indicates the removed trophectoderm cells.

It has been demonstrated that the biopsy protocol might affect clinical outcomes [36]. The approach entailing sequential hatching and biopsy results in a significantly higher survival rate after thawing, implantation, clinical pregnancy, and LB rate if compared to the cleavage stage hatching approach. However, day 3 pre-hatching, extends the time of exposure outside the incubator and the risk of having a blastocyst herniating from the inner cell mass requiring extra manipulation during the biopsy. Furthermore, this procedure allows a better synchronization with the natural expanding process of the blastocysts that could take place on day 5, 6, or 7. This technique is also cost-effective, since leaving the embryo undisturbed from fertilization to blastocyst formation allows for the employment of single-step media and time-lapse incubation protocol. Another controversial theme regarding TE biopsy is whether day 6 and day 7 blastocysts should be analyzed or not. A study by Piccolomini and co-workers [37] investigated if slow development might reflect embryo ploidy status. This group compared blastocyst biopsy performed on day 5 versus day 6 and reported similar aneuploidy rate (61.4% on day 5 vs. 69.9% on day 6). The study by Taylor et al. [38] evidenced that day 5 blastocysts had a significantly higher chance of being euploid than day 6 blastocysts (54.6% vs. 42.8%). Both of the studies concluded that blastocysts formed on day 6 and have the same chance of resulting in a live birth rate as those formed on day 5. The study by Hernandez-Nieto et al. [39] found that the rate of embryo euploidy was significantly lower in day 7 blastocysts when compared to day 5 or day 6 cohorts (40.5% vs. 54.7% vs. 52.9%, respectively). In his study there was also a significant decrease in the odds of implantation, clinical pregnancy, and LB, but no association with pregnancy loss in patients who transferred day 7 biopsied euploid blastocysts. Although day 5 blastocysts may have the higher euploid rates, its relationship with embryo development is still unclear [40,41]. On the other hand, day 7 blastocysts can be viable, of top morphology, euploid, and result in a healthy live birth. Therefore, culturing embryos an additional day increases the number of embryos useable per IVF cycle and provides further opportunity for patients who have only a few or low-quality blastocysts. These findings underlined the importance of performing biopsy of all blastocysts available independently of their morphology or growth-timing.

Non-invasive PGT

Embryo biopsy, performed at every developmental stage, is an invasive process that might condition IVF results. There are two alternatives to invasive biopsy: blastocentesis, consisting in the analysis of the blastocyst fluid (BF), and the examination of the spent culture media. The sampling of BF is performed on the opposite side of the inner cell mass, leaving the embryo fully collapsed [42,43]. Because dynamic collapse and re-expansion of the cavity is a phenomenon routinely observed during laboratory practice, the loss of the BF should not be detrimental to the embryo [44,45]. The aspiration of the BF does not affect embryo architecture, which results in high survival rates of both good and poor morphology embryos [46]. In 2013, Palini et al. [47], using real-time PCR, reported the presence of DNA fragments in BF obtained from day 5 blastocysts. The investigation of these DNA fragments allowed for the identification, with a 95% accuracy, of male embryos, detecting the specific Y-linked protein. Another study, conducted in 2015 by Tobler et al. [48], analyzed BF from 96 embryos: embryonic DNA was recovered and analyzed, using Whole Genome Amplification (WGA), followed by aCGH in 63% of the samples. The results were concordant with those of the matched inner cell mass karyotypes only in 48.3% of the analyzed embryos. This induced the authors to recommend not using blastocentesis as an alternative approach for PGT. Therefore, the failure of amplification rates after blastocentesis are a lot much higher if compared with those of the traditional TE biopsy [49]. On the contrary, Gianaroli et al. [50] reported the detection of embryonic DNA in 76.5% of the samples, with a diagnosis concordance rate of 97.4%, when compared to the correspondent TE biopsy. Al-

though the analysis of BF seems to be a promising alternative to invasive PGT, further studies must be conducted. It is important to establish whether the loss of the BF could alter cell to cell communication, or the communication of the embryo with its environment. Furthermore, it is still unknown if the DNA material obtained from the blastocentesis is representative of the embryo DNA.

PGT molecular techniques

The aCGH technique allows for detecting variations in the number of copies and rearrangements of each of the 24 chromosomes when comparing the biopsied genetic material with a reference sample. After amplification by WGA the sample is labelled with fluorescent probes and hybridized to a DNA microarray. The color adopted by each spot after hybridization allows for identifying chromosomal loss or gain. A laser scanner and a data processing software are used to detect fluorescence and analyse aneuploidy and chromosomal rearrangements [51-54]. Single Nucleotide Polymorphism Array (SNP) is performed using an array setup consisting in DNA hybridization, fluorescence microscopy, and solid surface DNA capture. SNP found in the analyzed sample are compared with SNP of maternal and paternal derivation to assess the ploidy status [55].

PGT in a good-prognosis patients undergoing SET

Choosing the best embryo to transfer is crucial, especially when a single embryo transfer program is adopted for different clinical reasons [56]. The first study to prospect a successful elective SET after a rapid on-site aCGH application was performed by Yang et al. [57] in good prognosis women <35 years of age. Fifty-six patients were randomized in two groups: in the first one a morphological evaluation of the embryos was used to select the one for the transfer in combination with aCGH, in the second one, morphology was used as the only discerning parameter. The aneuploidy rate in 425 blastocysts analyzed with aCGH was 44.9%, whereas 389 blastocysts were microscopically examined in the control group. The clinical and ongoing pregnancy rates were significantly higher in the morphology plus aCGH group as compared to controls (70.9 vs. 45.8%, and 69.1 vs. 41.7%, respectively). No twin pregnancies occurred in both groups. A low miscarriage rate was noted for all of the study patients, although this was slightly lower in the PGT-A group (2.6% vs. 9.1%). Despite an increasing acceptance of elective SET treatment, many IVF cycles continue to involve the transfer of two or more embryos. Scott et al. evaluated whether blastocyst biopsy with rtq-PCR comprehensive chromosomal screening might improve IVF outcome in women under 42 years with normal ovarian reserve. The aneuploidy rate was 28% among patients who were included in the genetic testing group. Clinical implantation rate and the proportion of screened embryos that progressed to delivery (79.8% and 66.4%, respectively) were significantly higher when compared to the control group (63.2% and 47.9%, respectively).

PGT for monogenic diseases

Pre-Implantation Genetic Testing for Monogenic Diseases (PGT-M) is an advisable approach for couples with the risk of transmitting genetic diseases to their offspring. However, chromosomal aneuploidies can involve chromosomes that different from those that were investigated with PGT-M. The first successful attempts to perform a double factor analysis (PGT-A and PGT-M) were reported by Obradors and collaborators [58]; the aim was to improve the implantation rate selecting potentially euploid embryos free of mutations responsible for cystic fibrosis [59] or Von Hippel–Lindau syndrome [60]. However, in both case reports, first genetic screening was performed by aCGH on oocyte polar bodies for PGT-A and the second using PCR on day 3 blastomeres for PGT-M. A similar procedure was applied by Rechitsky et al. [61] in 96 cycles resulting in the transfer of 153 unaffected aneuploidy-free embryos and 32 healthy live births. The value of this double screening was also explored by Goldman et al. [62] in a retrospective cohort study, including patients who underwent PGT-M with or without 24-chromosome aneuploidy screening. There were no differences between the PGT-M and aneuploidy screened group and PGT-M only group, when comparing the percentage of blastocysts affected by the single gene disorder of interest (37.0% vs. 32.8%).

Mosaicism

Mosaicism is defined as the presence of different cell lines in the same embryo. Two different kinds of mosaicism can occur: diploid/aneuploid mosaic with a mix of aneuploid and euploid cell lines and aneuploid mosaic with a mix of cell lines with different chromosomal abnormalities. There can be various types of aneuploidies in mosaic embryos: single chromosome loss or gain, complex or structural aneuploidies [63]. The origin of mosaicism is related to mitotic errors happening after fertilization at the third division stage. These mitotic errors, taking place before DNA duplication, are basically: anaphase delay, mitotic non-disjunction, accidental chromosome demolition, or premature cell division. The aneuploid cells rate depends on the time at which mitotic error happens; in embryos in which errors take place at the second cleavage stage, there will be a higher percentage of aneuploid cells [64,65]. Occasionally, mosaicism may derive from a meiotic non-disjunction event, causing a trisomic conceptus, followed by a post-zygotic event (trisomy rescue) [66,67]. Mosaic embryos have not been considered to be suitable for transfer and they were discarded, while considering them as aneuploid embryos. Mosaicism was supposed to be responsible for altered embryo development, thus leading to implantation failure, or resulting in congenital malformation, mental retardation, and uniparental disomy [68].

Mosaic blastocysts transfer

Implantation is considered to be an essential step for the success of assisted reproduction techniques and mainly depends on endometrial receptivity, embryo quality, and synchrony between them. However, the process of ovarian stimulation with elevated estrogen level, together with a possible progesterone premature growth, might reduce the expression of genes involved in the implantation process and negatively modify embryo-endometrium communication [69]. These negative effects can be responsible of decreased clinical results and adverse obstetrics and perinatal outcomes. It has been suggested, indeed, that, after a fresh embryo transfer in a stimulated IVF cycle with E2 levels >2724 pg/mL at the time of hCG ad-

ministration, the risk of abnormal placentation and low birth weight [70] as well as the risk of obstetric hemorrhage [71] is definitely higher.

Conclusions

The PGT is a valid technique to evaluated embryo euploidy and mosaicism before transfer. Next generation sequencing is considered by several studies as the best molecular test and trophoectodermal biopsy at the blastocyst stage is today the most used method for embryo biopsy. Preimplantation genetic testing is currently under study for assessing its usefulness, safety, and clinical validity. The clinical application of PGT-A are mainly those conditions in which the risk of embryo aneuploidies might increase, such as advanced maternal age, recurrent pregnancy lost, repeated implantation failure, severe male infertility factor, or when a single embryo transfer is necessary. The clinical benefit of this strategy in good prognosis patients and egg donation programs should be assessed by properly designed randomized control trials, especially if single embryo transfer is requested. Maternal and neonatal outcomes seem to be reassuring but more studies are needed. Mosaic embryo should be considered for transfer after an appropriate genetic counseling for the transfer for patients without euploid embryos.

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