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Clinical application of embryo aneuploidy testing by next-generation sequencing

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Abstract

We review here the evolution in the field of embryo aneuploidy testing over the last 20 years, from the analysis of a subset of chromosomes by fluorescence in situ hybridisation to the transition toward a more comprehensive analysis of all 24 chromosomes. This current comprehensive aneuploidy testing most commonly employs next-generation sequencing (NGS). We present our experience in over 130 000 embryo biopsies using this technology. The incidence of aneuploidy was lower in trophectoderm biopsies compared to cleavage-stage biopsies. We also confirmed by NGS that embryo aneuploidy rates increased with increasing maternal age, mostly attributable to an increase in complex aneuploid embryos. In contrast, the number of MII oocytes retrieved or the use of oocyte vitrification did not affect aneuploidy rates. Similarly, neither maternal age, oocyte number, nor oocyte vitrification affected the incidence of mosaicism. Analysis of clinical outcomes, indications, and potential benefits of embryo aneuploidy testing revealed advanced maternal age as the most favored group, with some evidence of improved delivery rate per transfer as well as decreased miscarriage rates and time to pregnancy. Other indications are: recurrent miscarriage, repetitive implantation failure, severe male factor, previous trisomic pregnancy, and good prognosis patients mainly undergoing single embryo transfer, with the latter indication used to reduce the occurrence of multiple pregnancies without compromising cycle outcome. In conclusion, NGS has become the most appropriate technology for aneuploidy testing in trophectoderm biopsies, with accurate results, high throughput, and cost efficiency. This technology can be also applied to the analysis of the embryonic cell free DNA released to the culture media at blastocyst stage. This is a promising approach towards a non-invasive preimplantation genetic testing of aneuploidy.

Summary Sentence

NGS has become the most appropriate technology to apply for aneuploidy testing in trophectoderm biopsies, offering accurate results with a high throughput and cost efficiency.

Key words: Embryo, blastocyst, aneuploidy, trophectoderm biopsy, NGS.

Introduction

The most common genetic abnormality identified in human embryos is aneuploidy. This abnormality is particularly common among embryos produced by in vitro fertilization (IVF)—more than half are aneuploid [1]. Hence, analyzing chromosomal status of the embryo using preimplantation genetic testing for aneuploidy (PGT-A) enables embryo selection that can result in better ongoing pregnancy rates per transfer in infertile couples requiring assisted reproductive technology (ART).

However, the application of PGT-A raised controversy after the publication of several randomized controlled trials (RCT) using FISH [2]. The concerns were mainly due to technological limitations that only allowed analysis of a small number of chromosomes and inherent issues analyzing FISH signals on a single cell biopsied from an embryo containing 6–8 cells [3]. These concerns led to the development of new diagnostic technologies offering the ability to interrogate all 23 chromosome pairs, including single nucleotide polymorphism (SNP) arrays, quantitative polymerase chain reaction (qPCR), array comparative genome hybridization (aCGH), and, more recently, next-generation sequencing (NGS). NGS is now the most commonly applied technique for PGT-A

PGT-A using more comprehensive techniques have improved embryo and clinical outcomes as will be described later in the results section. Besides, NGS has allowed the identification of mosaic aneuploidies in trophectoderm (TE) biopsies with different levels of detection. Despite these technological advances, it remains challenging to calculate the real incidence of mosaicism in preimplantation embryos due to technical and biological limitations. A TE biopsy represents a small percentage of total number of cells of the embryo and will only partially represent the whole blastocyst, depending on percentage and distribution of euploid and aneuploid cells in the inner cell mass and TE. NGS can detect mosaicism at lower frequencies in TE biopsies than previous technologies. However, it is important to validate each NGS platform for accurate detection of low-degree mosaicism and differentiate from experimental noise related to the quality and quantity of biological samples and amplification artifacts. There is consensus among most groups to report mosaicism in >30% of estimated aneuploid cells. In addition, customized software and algorithms are being developed to improve robustness and objectivity among observers to identify mosaicism.

Recent studies either retrospective or based on the observation of clinical outcomes following a highly selected cohort of mosaic embryos have proposed the possibility of transferring some types of mosaic embryos. However, most show lower implantation and higher miscarriage rates than the transfer of euploid embryos [4, 5, 6]. Further research is needed to understand the relevance of mosaicism in TE, as there are no studies on the effects of different percentages of aneuploid cells in the blastocyst and affected chromosomes. Vera-Rodriguez & Rubio [7] presented a review of the topic, and a recent study by Grati et al. added some insights on the risk of transferring mosaic embryos for each chromosome. They developed a scoring system to prioritize the transfer of mosaic aneuploid embryos, considering that individualized genetic counseling is crucial [8].

The purpose of this study is to present updated results on the detection of aneuploidy by NGS in day-3 and TE biopsies, and to discuss current literature on the clinical outcomes of comprehensive aneuploidy testing.

Current experience in PGT-A using NGS

New technologies have facilitated the transition from FISH analysis of a limited number of chromosomes to analysis of all 23 chromosome pairs simultaneously in a single cell. Among these technologies, results of qPCR, SNP arrays, and aCGH have been most widely published to date; these approaches have been applied to polar bodies and day-3 and TE biopsies [9]. Array comparative genome hybridization technology allows analysis of chromosome DNA copy number variations from an embryo compared to a reference sample. First, DNA from a single blastomere or 4–6 TE cells is amplified via whole genome amplification (WGA). Amplified DNA is then labeled with different fluorescent probes, combined, and hybridized onto a slide containing specific bacterial artificial chromosome probes that span the length of chromosomes with ~1 Mb coverage. Chromosome loss or gain is revealed by the color of each spot after hybridization. Fluorescence intensity is detected using a laser scanner and data processing software, which can analyze whole chromosome aneuploidy and sub-chromosomal structural imbalances [10]. SNP arrays also utilize an array setup, although they interrogate specific SNPs in the genome and compare these data to SNP patterns of maternal and paternal origin to arrive at a ploidy call [11]. For qPCR, specific PCR primers amplify a limited section of each chromosome on all 23 chromosome pairs in replicates. By analyzing the relative amount of DNA from each PCR product, a ploidy status can be inferred and assigned to each chromosome [12].

The latest approach for comprehensive aneuploidy testing is NGS. For NGS, most extended protocols share the first steps with aCGH protocols, starting with WGA. In NGS, a barcoding procedure follows WGA in which different samples are labeled with unique sequences, so that they later can be mixed, sequenced, and matched to their original patient and/or embryo. This barcoding process allows 24–96 biopsies to be pooled in a sequencing run depending on the sequencing platform, optimizing cost per sequenced embryo. After sequencing, each sequence is aligned with a reference human genome, and copy number variations for whole chromosomes and large deletions/duplications (del/dup) are established using specific software [13–16]. Figure 1 shows the profiles of the following categories: a–b) euploid female and male; c) single aneuploidy (one aneuploid chromosome); d) complex aneuploidy (2–5 aneuploid chromosomes); e) chaotic pattern (>5 aneuploid chromosomes); or f) segmental aneuploidy (duplications/deletions >10–15 Mb) (Figure 1).

To determine mosaicism levels in TE biopsieseach laboratory should define their proper thresholds to differentiate among several categories of mosaicism. After this validation, in our laboratory we established two types of mosaicism: I) low degree of mosaicism, 30– <50% estimated aneuploid cells; and II) high degree of mosaicism, 50– <70% aneuploid cells. Figure 2 shows profiles with different levels of mosaicism. There have been publications from different authors establishing different criteria for the definition of mosaicism. Fragouli et al. [5] validated mosaicism for different ranges of aneuploid cells from 20–80% and Spinella et al. [6] from 10–90% aneuploid cells. In the latest one, two different categories were established: low mosaic (<50% aneuploidy) and high mosaic (≥50% aneuploidy), with different prognosis if transfer was attempted.

Below, we present the results from 133 545 biopsies analyzed by NGS in 2016 and 2017. Results are presented according to biopsy type; we assessed 3525 cycles with cleavage-stage biopsy and 30 770 cycles with TE biopsy.

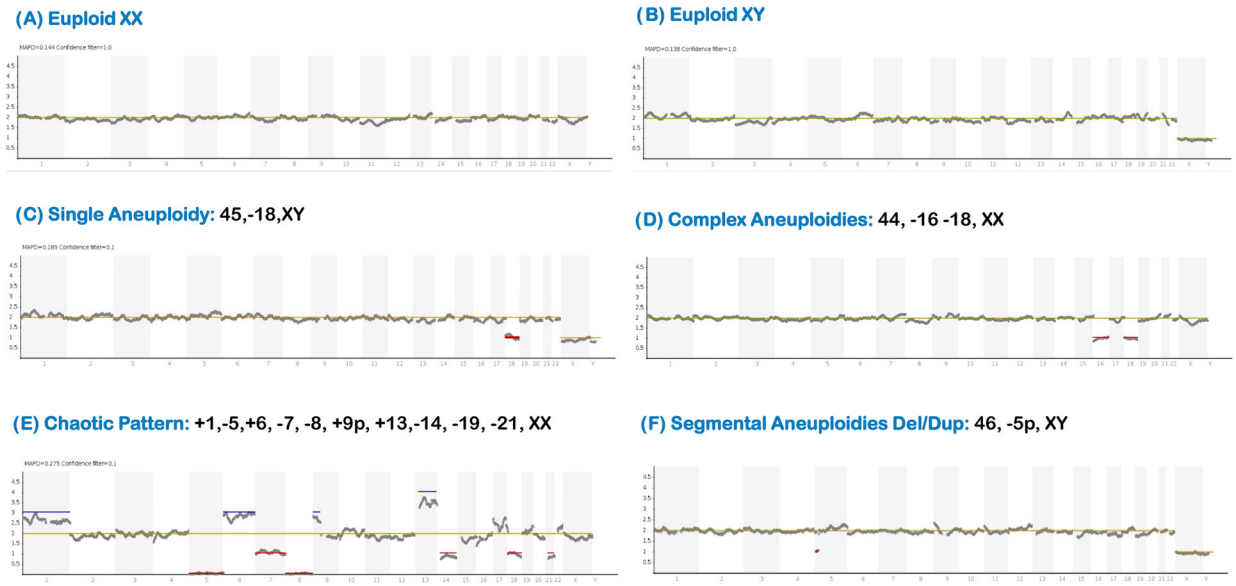

Figure 1. Classification of embryo biopsies analyzed by NGS: a–b) euploid female and male; c) single aneuploidy (one aneuploid chromosome); d) complex aneuploidy (2–5 aneuploid chromosomes); e) chaotic pattern (> 5 aneuploid chromosomes; and f) segmental aneuploidy (duplications/deletions >10–15 Mb).

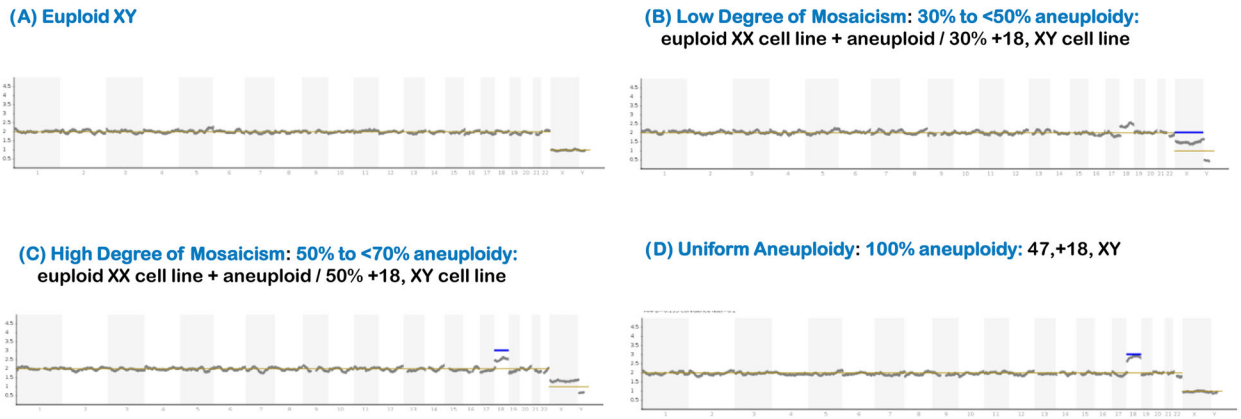

Figure 2. Representation of the different levels of mosaicism reported with NGS in validation studies mixing cell lines with different karyotypes: a) euploid; b) low degree of mosaicism: 30%– <50% aneuploidy; c) high degree of mosaicism: 50%– <70% aneuploidy; and d) uniform aneuploidy: 100% aneuploidy.

Aneuploidy rates detected by NGS are presented in Figure 3 according to the biopsy strategy and day of biopsy. There was a significant decrease in the incidence of aneuploidies in TE compared to cleavage-stage biopsy, mainly for day-5 and day-6 TE biopsies. This decrease could be explained by the fact that TE biopsies show significantly lower percentages of chaotic aneuploidies compared to cleavage-stage biopsies, but other alternative explanations such as technical artifacts when analysing a single cell versus multiple cell biopsies with a potential overestimation of chaotic biopsies and biological factors should be taken into account.

Results of 106 960 TE biopsies are presented in Figure 4 according to maternal age as well as from egg donors. Maternal age ranged from 19–44 years. Overall aneuploidy rates increased according to maternal age, as expected. Abnormalities that were significantly more frequent in older women were complex abnormalities with 2–5 aneuploid chromosomes. This implicates poorer meiotic segregation fidelity as women age. However, the percentage of del/dup and other types of aneuploidies do not reflect a maternal age effect.

We also analyzed a subset of 6295 TE biopsies in women <38 years of age according to the number of MII oocytes (Figure 5). No significant correlation was observed between the ovarian response and aneuploidy rates, with a slight linear decrease in the percentage of complex aneuploidies.

In a subset of 5849 TE biopsies in women 20–44 years of age, we were able to apply our customized algorithm to determine the incidence of high and low degrees of mosaicism. We observed an overall mosaicism rate of 6.7%, with 4.3% being low-mosaic aneuploid embryos and 2.4% high-mosaic aneuploid embryos, according to our internal classification. Maternal age did not appear to affect mosaicism rates, though we observed a slight decrease in women >37 years of age compared to the younger group, including ovum donation (4.3 vs. 7.0%). There was no significant relationship between mosaicism and the number of MII oocytes (5.5 vs. 7.2%) in women with ≤15 compared to women with >15 MII oocytes.

Finally, oocyte vitrification did not increase the overall abnormality rates (59.3 vs. 58.7%) or mosaicism rates (6.0 vs. 6.9%) when

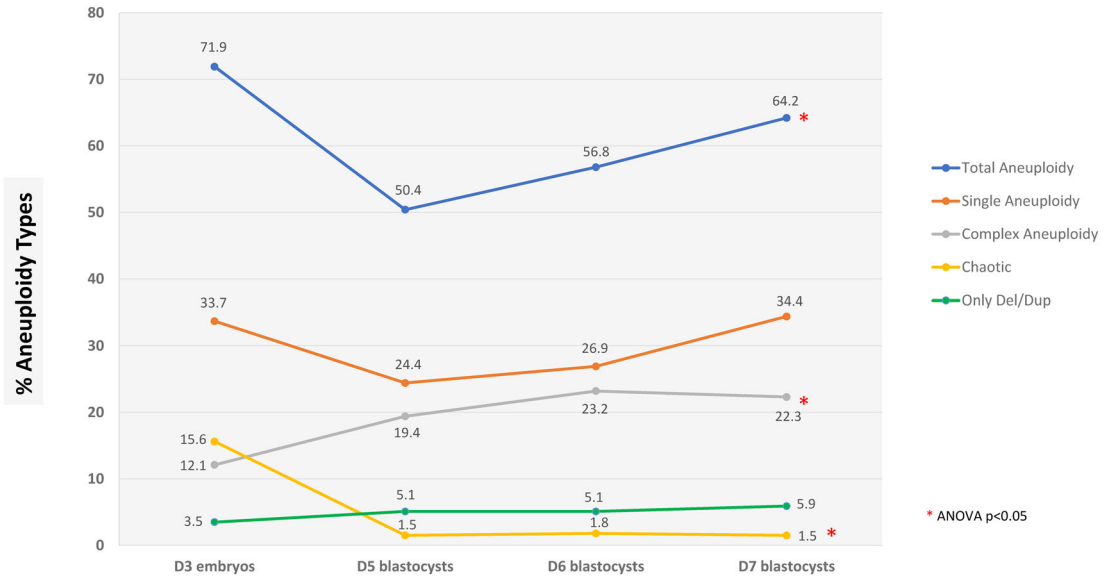

Figure 3. Incidence of different aneuploidy types according to the day of embryo biopsy.

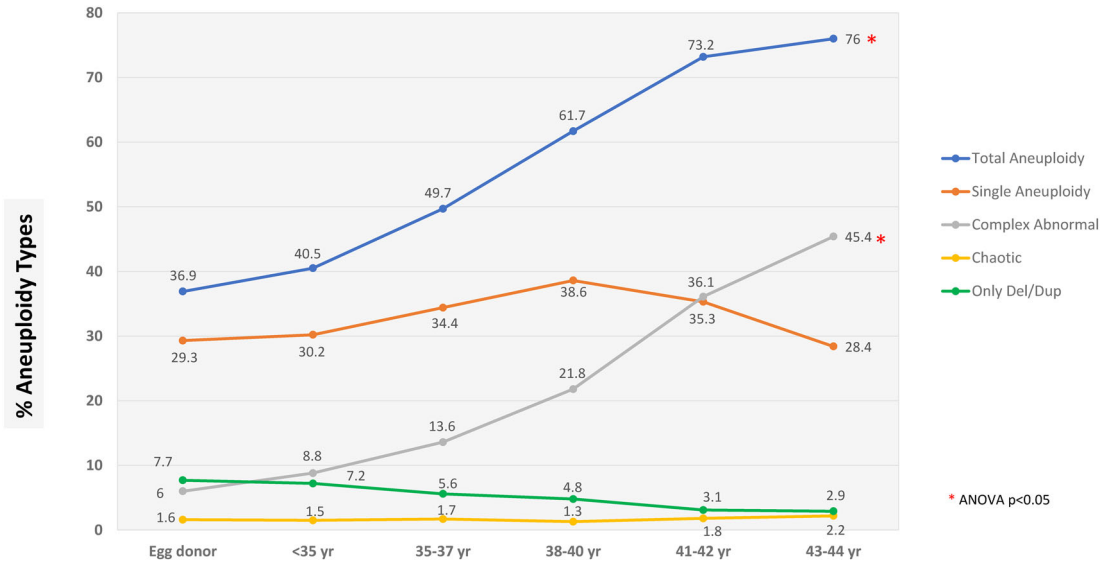

Figure 4. Incidence of different aneuploidy types in donated oocytes and in own oocytes related to maternal age.

compared to fresh oocytes in a subset of 4137 TE biopsies in which the origin of the oocytes was detailed.

The main goals for most indications of PGT-A are not only to increase implantation and pregnancy rates, but also to decrease miscarriages, risk of aneuploid offspring, and time to conceive. More recently, cost efficiency per healthy baby at home also has been considered, because with new PGT-A 3.0 (blastocyst biopsy and NGS), cost is no longer a limitation for the implementation of embryo aneuploidy testing [17].

Further, with the increasing popularity of blastocyst biopsy, blastocyst vitrification after TE biopsy, and deferred transfer, PGT-A fits well into this clinical scheme. A recent study comparing fresh blastocyst transfer and frozen cycles shows improved implantation rate per transferred embryo (75 vs. 67%), although the difference is not statistically significant. However, ongoing pregnancy rates (80 vs. 61%) and live birth rates (77 vs. 59%) are significantly higher with frozen samples compared to fresh transfer. Either transfer strategy can be a reasonable option, but there is a trend toward favuring deferred transfer [18]. NGS has also shown improvements in clinical outcome compared to aCGH in couples undergoing single embryo transfer (SET) [19].

PGT-A indications

The following are the most common current indications for PGT-A:

Advanced maternal age

Advanced maternal age (AMA) is the most common indication for PGT-A. Maternal age is a major factor in the prevalence of aneuploidy. Most clinical IVF groups have traditionally considered AMA

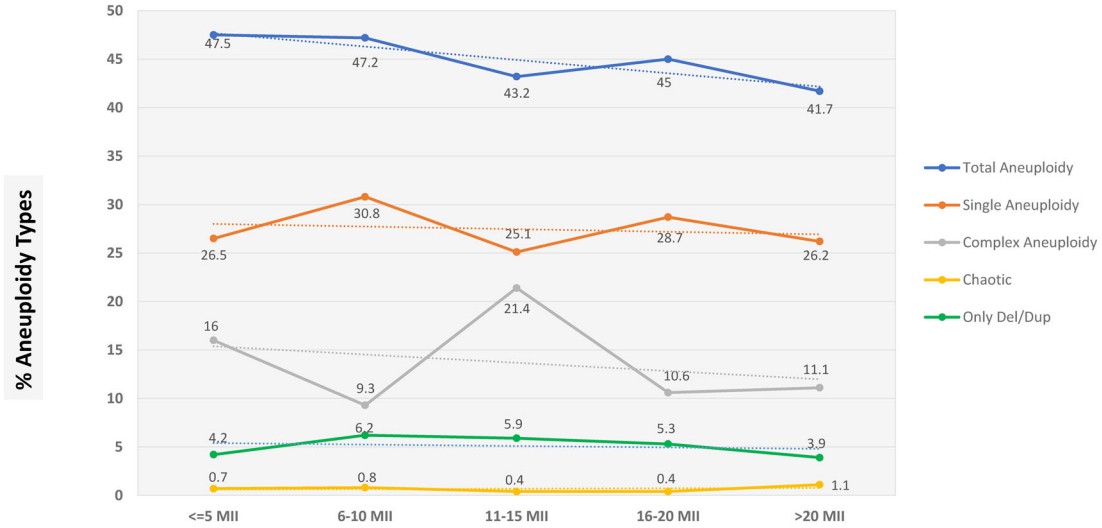

Figure 5. Incidence of different aneuploidy types in patients with different numbers of MII oocytes retrieved.

to be any patient >37 years old, although recently there is a move to lower this to 35 years.

A systematic review and meta-analysis of four RCTs indicates that PGT-A offers no benefit to AMA patients [2]. However, our own experience differs from previously published studies. We conducted two prospective, randomized trials to evaluate the usefulness of PGT-A in AMA patients, the first using PGT-A-FISH in women 41–44 years of age. In this study, we observed a significant increase in live birth rates in the PGT-A-FISH group compared to the conventional blastocyst transfer group (32.3 vs. 15.5%; $p = 0.0099$). We therefore concluded that classic PGT-A 1.0 was beneficial [20]. Despite these results, there remained a need for a technique to analyze all chromosomes while also producing reliable and accurate results in a short period of time. Therefore, a second study using aCGH in women 38–41 years of age confirmed higher live birth rates using PGT-A compared to conventional morphological embryo selection per first transfer (52.9 vs. 24.2%; $p = 0.0002$) and per patient (36.0 vs. 21.9%; $p = 0.0309$). Notably, PGT-A dramatically decreased miscarriage rates compared to controls (2.7 vs. 39.0%) [17].

Without the ability to screen for aneuploidy, AMA patients with a high percentage of aneuploid embryos may be subjected to multiple unsuccessful embryo transfers for months, some of which may end in miscarriages (and the associated medical risks) or in live birth of a child with a chromosomal abnormality.

Recurrent miscarriage

The definition of recurrent miscarriage (RM) varies by country, but it is generally considered the occurrence of 2–3 consecutive miscarriages with a gestational age up to 14 weeks. For PGT-A, other causes of miscarriage should be discarded with a proper infertility work-up before indicating this treatment. However, there is increasing evidence supporting the use of PGT-A. A recent study compiling the results of prenatal diagnosis in 46 939 women confirms an increased risk of karyotypic abnormalities in conception products of idiopathic RM patients [21]. Our studies demonstrate that, after PGT-A, couples who previously suffered aneuploid miscarriages have significantly higher implantation rates and lower miscarriage rates. We also concluded that PGT-A should be recommended when RM is associated with a previous aneuploidy and when there is a high incidence of chromosomal abnormalities in sperm [22]. A systematic review also suggests that PGT-A may lower miscarriage rates [23]. A retrospective case-control study reports PGT-A implantation rates of 52.63% compared to 19.15% in controls ($p = 0.001$) and an almost doubled ongoing pregnancy rate (61.54 vs. 32.49%; $p = 0.0001$) [24]. Further, another study comparing clinical outcome in PGT-A after day-3 or TE biopsies reports ongoing clinical outcomes of 50.4 and 63.6%, respectively [25].

Repetitive implantation failure

Repetitive implantation failure (RIF) is defined as three or more failed IVF attempts or failed IVF treatments after cumulative transfer of >10 good-quality embryos. RIF-defining criteria are not homogenous, and an exhaustive and comprehensive definition has not yet been reached. Therefore, RIF remains a challenge to clinicians because it can have multiple causes that are poorly defined.

One RCT in RIF patients concluded that there were no significant differences in clinical pregnancy rates with PGD-A-FISH compared to controls [26]. However, another study that analyzed a few more chromosomes showed a clear trend toward better live birth rates with PGT-A-FISH (47.9 vs. 27.9%) [20].

Further, an aCGH study including 467 RIF couples showed that different factors affect clinical outcomes. In day-3 biopsies, pregnancy rates are 52.6% in patients <40 years old, compared to 41.5% in older patients. The best prognosis is observed in younger patients with a sperm concentration of <10 million sperm/mL and with >15 mature (MII) oocytes. Number of previous failed cycles only increases the probability of couples producing embryos with a complex division pattern, but it does not impact their overall clinical implications. In a subset of patients with TE biopsies and deferred blastocyst transfer, pregnancy rates per transfer are 73.3% [27].

Severe male factor infertility

An increased incidence of chromosome abnormalities has been reported in sperm samples of infertile men with normal FISH karyotypes, with oligozoospermia associated with significant increases in sex chromosome disomy, chromosome 18 and 21 disomy, and percentage of diploid sperm, particularly in samples with markedly reduced sperm concentrations ($<5 \times 10^6$/mL spermatozoa). Such

conditions might, in part, explain low implantation and high miscarriage rates observed in these patients [28].

Testicular sperm from non-obstructive azoospermia and from carriers of Y-microdeletions also show increased sperm aneuploidy, mostly for sex chromosomes [29, 30]. Different types of sperm chromosomal aneuploidies are translated in the embryos, following a similar pattern, with increased trisomy for sex chromosomes in embryos from sperm samples with increased sex chromosome disomies, and higher triploidy rates in embryos from sperm samples with increased diploidy rates [31].

Blastocyst biopsies have significantly greater sex chromosome abnormalities compared to embryos derived from normal semen samples. Further, aneuploidy rates in embryos derived from sperm with normal parameters are not significantly different whether intracytoplasmic sperm injection or standard insemination is used to achieve fertilization. These results highlight severe male factor infertility as a possible referral category for PGT-A [32]. Interim analysis of an RCT with day-3 biopsy and aCGH in couples with $<2 \times 10^6$ sperm observed increased ongoing pregnancy and implantation rates, suggesting severe oligozoospermia as an indication for aneuploidy testing [33].

Previous trisomic pregnancy

Some studies suggest that a previous trisomic pregnancy is associated with increased risk of another aneuploid conception. A study comparing the rates of aneuploidy in preimplantation embryos from women with a history of a previous aneuploid conception concluded that a history of trisomic pregnancy, whether or not it was a viable trisomy, is associated with increased risk of another aneuploid conception [34]. In 2009, De Souza and colleagues [35] used data from an Australian population-based birth defects registry to establish whether the risk of trisomies 13, 18, and 21 (Patau, Edwards, and Down syndrome, respectively) in a subsequent pregnancy was higher for women who have had a previous pregnancy with trisomy 13, 18, or 21. The relative risk of a trisomy 21 pregnancy following a previous trisomy 21 pregnancy is greater for women <35 years old at the time of the previous pregnancy, as is the risk of the same trisomy and of a different trisomy subsequent to trisomy 13 or 18. Relative risk of a different trisomy subsequent to trisomy 21 is similar for women <35 and >35 years old at their previous pregnancy. The authors concluded that women who have had a previous trisomic pregnancy, particularly those <35 years old at the time, appear to have an increased risk of future trisomic pregnancies.

In relation to previous data, a more recent study stated that the incidence of chromosomal abnormalities in preimplantation embryos associated with a previous aneuploid miscarriage is significantly higher in individuals with a previous aneuploid conception [36]. In conclusion, the data suggest that using PGT-A can avoid recurrence of aneuploidies and could benefit this group of patients.

Good-prognosis patients and single embryo transfer

For patients with a good prognosis, it was proposed that TE biopsy with aneuploidy testing for 24 chromosomes could have high potential to increase overall pregnancy rates in IVF programs and to decrease multiple pregnancies when SET is performed. The first RCT comparing blastocyst-stage SET with and without PGT-A in good-prognosis patients showed an aneuploidy rate of 44.9% among biopsied blastocysts, with a significantly higher clinical pregnancy rate in the PGT-A group (70.9 vs. 45.8%; $p = 0.017$) and no twin pregnancies. This study reveals the limitations of SET when conventional morphology is used alone, even in patients without an increased risk for aneuploidy, with greater efficiency and yielding a lower miscarriage rate in the PGT-A group [37].

Two subsequent RCTs compared PGT-A with routine IVF techniques in good-prognosis patients undergoing ART. In the first trial, patients in the study group underwent euploid blastocyst SET, whereas the control group underwent blastocyst double embryo transfer (DET) based only in morphology. Clinical outcomes include a similar ongoing pregnancy rate between groups (60.7% after SET vs. 65.1% after untested DET; 95% CI: 0.7–1.2) and reduced risk of multiple gestations after SET (48–0%) [38]. In the same year, another trial with infertile couples showed delivery rates per cycle were significantly higher in the PGT-A group ($p = 0.01$) [39].

More recently, a single-center retrospective study assessed the use of PGT-A in cycles with donor eggs and frozen embryo transfers using aCGH or NGS. Data were analyzed separately for SET, DET, and for own uterus and gestational carrier uterus recipients. In DET, the PGT-A group had significantly higher live-birth implantation rates, but not live birth rates per transfer cycle. In SET, PGT-A had nominally, but not significantly, higher live birth implantation rates and live birth per cycle compared to controls. This study provides preliminary evidence that application of PGT-A may improve IVF outcomes using younger oocytes from an egg donation cycle [40].

Non-invasive preimplantation aneuploidy testing

In PGT-A, the full chromosome content of a single or few cells is analysed with high sensitivity and specificity. However, the input material is obtained by an invasive method: a biopsy. Therefore PGT-A involves a high investment for the IVF laboratory in specialized equipment for the biopsy, such as the laser. In addition, this procedure must be conducted by trained embryologists to avoid operator-dependent bias in the results and to ensure the minimal impact on embryo viability. Recently, niPGT-A with the analysis of the embryonic cfDNA released to the culture media has been proposed as an alternative to overcome embryo biopsy for aneuploidy testing. Several studies have compared the results of PGT-A in TE biopsies with the results of the spent culture media, to establish the concordance rates among both approaches. Concordance rates were highly variable among the published studies, with a range from 3.5–85.7% [41–45]. The discrepancies in the reported results could be related to the different methodologies applied. How the embryo is handled during the whole process is extremely important because it can determine not only the quantity and quality of the DNA present in the spent culture medium, but also the presence of residual cumulus cells not completely removed from the oocyte that could lead to contamination with maternal DNA resulting in false negatives. Also the NGS protocol have to be modified from the one employed in PGT-A to achieve higher DNA amplification yield and high informativity rates. We have recently presented a study showing the informativity and concordance rates of TE biopsies with the analysis of the cfDNA in the culture media and the results have been encouraging, with high informativity rates close to 100% when the blastocyst media were collected after 48 h in culture and with 84.0% concordance rate with the TE biopsy [46].

Conclusions

NGS has become the most appropriate technology for aneuploidy testing in TE biopsies. This technique offers accurate results, comparable to those obtained with aCGH, with a high throughput and cost

efficiency. NGS can be also applied to the analysis of the embryonic cfDNA, by analyzing the spent culture avoiding the embryo biopsy. This approach will need further studies, but preliminary results are encouraging. Regarding clinical outcome for different indications, there is evidence of improved delivery rates per transfer for patients of AMA, comparing PGT-A vs. non-PGT-A blastocyst trnasfer, with decreased miscarriage rates and time to pregnancy Despite, cumulative pregnancy rates r were not different, we should bear on mind the decrease of both miscarriage rates and the number of transfers to achieve a livebirth, and therefore the time to pregnancy. In young patients with SET an improvement in ongoing pregnancy rates per transfer has been also reported, without data analyzing cumulative pregnancy rates. For other indications, more RCTs are needed, despite the published retrospective data indicating lower miscarriage rates and potential improvement of pregnancy rates.

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