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Review Article

Preimplantation Genetic Testing for Aneuploidies (PGT-A): Baring Our Limitations

C Fatemi[1], B Ata[2,1], I Elkhatib[1] and D Nogueira[3,1]*

[1]In-Vitro-Fertilisation (IVF) Department, ART Fertility Clinics, United Arab Emirates

[2]Department of Obstetrics and Gynecology, Koc University School of Medicine, Istanbul, Turkey

[3]Inovie Fertilité, France

***Corresponding Author:** D Nogueira, *In-Vitro*-Fertilisation (IVF) Department, ART Fertility Clinics, United Arab Emirates, Inovie Fertilité, France.

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Abstract

The clinical value of PGT-A remains an area of debate. Most data produced so far demonstrate that the intervention of PGT-A is safe since they neither support nor refutes its efficacy. The clinical value of a test rests in its validity. A fair assessment and interpretation of results based on the original experimental design are key determinants of the accuracy of the reported outcomes. A significant issue concerning non-inferiority trials on PGT-A are flawed experimental set ups which invalidate many of the conclusions. We highlight and challenge some of these reports herein. Moreover, other relevant aspects related to the PGT-A reports concerns the discernment between technical and true biological mosaicism. This critical distinction is essential since reporting mosaicism may primarily come at the expense of euploid diagnoses. Other significant aspects impacting PGT-A outcomes are specifically related to the embryology laboratory. Technical manipulations for PGT-A need validation as well proved standardized results in order to substantiate clinical performances. These factors range from type of blastocysts biopsied, choice of biopsy procedure and individual performance. Overall, the controversies on the efficacy of PGT-A are, in part, due to the methodological limitations of studies which fail to consider the influence of the technical aspects on the outcomes.

Keywords: *Preimplantation Genetics Testing for Aneuploidy (PGT-A); Trophectoderm Biopsy; Blastocyst; NGS; RCT*

How far, or limited, are we?

The field of IVF is recognized by its rapid innovations, leading to continuous efforts to investigate effectiveness of 'add-on' technologies. Accordingly, to offer best clinical practice, the existence of a combination of tools are required to identify the embryo with best potential for implantation. In fact, more than forty years after the birth of the first IVF baby, very few of the assisted reproductive technologies can nowadays be identified as well-established treatments in routine use, such as ICSI and IVF. Pre-implantation genetic testing for aneuploidies (PGT-A), is still considered as an add-on with controversial outcomes in relation to its effectiveness, as exposed by the RCTs so far published.

Genetic technologies are in constant development from diagnostics to therapeutics in the medical field and have been for years integrated as a tool for embryo selection in IVF. Historically, the revolutionary PGT-A technology evolved from "PGT 1.0", i.e. identifying aneuploidy on blastomeres of day 3 embryos analysed by fluorescence *in situ* hybridization (FISH), moving to trophectoderm (TE) cells

on day 5, “PGT 2.0” with the advances in genetic analyses screening using array-comparative genomic hybridization (a-CGH) followed by the contemporary PGT-A, the “PGT 3.0” applying next-generation sequencing (NGS). The earliest methodologies failed to demonstrate an increase in live birth rate as exposed in 13 RCTs [1]. The latest methodology derived improvements in the implantation rate per embryo transfer, reduced miscarriage rates, number of embryo transfer procedures and time to live birth [2], along with two recent non-selection studies demonstrating clearly the absence of a live birth when a full chromosome aneuploidy is observed [3,4]. While the clinical error rates for the commonly used PGT-A techniques have been ≤ 2% [5-8], the quality of evidence suggesting improved clinical outcomes with the “PGT 3.0” is still low, due to the limited number of RCTs and with technical heterogenicity.

Currently, there is an ongoing debate of which patients could benefit from PGT-A, whether to only apply PGT-A for certain patient profiles, e.g. advanced maternal age (AMA), severe male factor, recurrent implantation failure (RIF) and recurrent pregnancy loss (RPL), or to apply PGT-A to a broader patient population. However, prior debating on those patient related questions, one should not neglect the technical challenges leading to misleading clinical decisions, which will be highlighted in this paper.

Challenging the so-called evidence-base

A recurrent issue relates to the appraisal of PGT-A reports. A fair assessment and interpretation of results based on the original experimental set up are key determinants of the accuracy of the reported outcomes. For instance, the recent RCT published by Yan., *et al*. [9] showed a non-inferior cumulative live birth rate in conventional IVF (77.2%) compared to PGT-A group (81.8%). However, the authors included only younger than 38-year-old patients with good prognosis and with Trophectoderm (TE) biopsies performed solely in a selective pool of three blastocysts of best morphology. In fact, few relevant points must be considered when carrying out RCTs on PGT-A in relation to the choice of embryos to biopsy. Firstly, blastocyst phenotype evolves rapidly, even possibly from a ‘bad-to-good’ morphology and vice-versa, within a short lag of time. The authors’ choice to biopsy only a restricted number of embryos on day 5 may be considered therefore a bias, introducing disadvantage to the PGT/A arm. The currently available literature tends to favour PGT-A over embryo morphology for selection. Therefore, choosing a restricted number of blastocysts for biopsy impacts on the euploidy rates and on the number of patients with euploid embryos available for transfer in PGT-A group. Consequently, a portion of patients dropped out of PGT-A arm in the RCT by Yan., *et al*. [9] due to the absence of euploid embryos among the three biopsied blastocysts. Secondly, authors only biopsied embryos that reached blastocyst stage by day 5 of development.

However, currently available evidence confirms that day 6 blastocysts have considerably high chances of being euploid [10,11]. It is well possible that a patient from whom embryos biopsied on day 5 are aneuploid, have euploid embryos biopsied on day 6. Thirdly, not all embryos classified as good morphology are “ripe” for biopsy. Experienced teams acknowledge that the aptitude to homogenize the selection of the type of embryos submitted for biopsy render results of highest accuracy. This means that if one needs to limit the number of “good-looking” blastocysts for PGT-A to fit a RCT design, it is possible that a blastocyst composed of a more robust TE might be chosen over a “better-looking” blastocyst but constituted of fewer TE cells. Alternatively, one might choose the “better-looking” blastocyst of the cohort but with fewer TE cells. In this case, the TE biopsy may lead to a misleading or non-informative genetic result in view of reduced DNA/cell content. Best practice incites us to perform biopsies in all usable blastocysts, and in function of TE cells proliferative aspect, even if the biopsy procedure must be carried out in different times for the same patient. Another point that merits reflection on the interpretation of studies, such as the RCT by Yan., *et al*. [9], is the fact that genetic analysis is performed using multiple genetic platforms rather than using a single platform. This approach is likely to lead to a discordance in mosaic and normality rates among the platforms [12,13] and thus on the euploidy outcomes. Similar approach was taken in the “STAR trial” [14]. This RCT intended to assess the benefit of PGT 3.0 with NGS, and included a total of 661 patients aged between 25 and 40 years with at least two biopsied blastocysts [14]. They observed no difference on ongoing pregnancy rate per embryo transfer between PGT-A (50%) and non-PGT-A (46%), and neither on intention to treat per protocol, 41.8% compared to 43.5%, respectively. However, a higher ongoing pregnancy rate (OPR) per embryo transfer was observed in PGT-A (51% versus 37%) in women aged 35 - 40 years. Miscarriage rates were about 10% in all age groups. Authors concluded

that for women under the age of 34, PGT-A is not as effective and did not decrease miscarriage rates in these good prognosis patients [14]. As this was a multicentre RCT including 34 different IVF clinics and 9 different genetic laboratories in four different countries, it is subjected to discrepancies in results. As the authors stated, "each clinic followed their own standard of care regarding ovarian stimulation, endometrial preparation, luteal-phase support and IVF laboratory procedures". Furthermore, the 16.8% of the PGT-A embryos that were classified as mosaic, are far above the acceptable frequency (< 5%) [15]. Moreover, only five laboratories reported mosaic embryos as "no aneuploidy detected" or "abnormal", which is incorrect since transfer of "mosaic" blastocysts in the absence of an euploid alternative is already the recommended approach since 2016 (Preimplantation Genetic Diagnosis International Society 2016, Practice Committee and Genetic Counselling Professional Group, 2020). This means that a considerable proportion of embryos could have been euploid and might have distorted the outcome reported [16]. Authors themselves call for the importance of a prerequisite for optimization and validation of laboratory procedures [14], which might result in differences in mosaicism rates. This includes both IVF (embryo biopsy and vitrification) and genetic testing procedures [17].

Stimulation cycle management in the IVF clinics have also been suggested as a potential source for variability in genetic outcomes. Munne., *et al*. [14], by using a single genetic laboratory performing PGT 2.0, reported up to 40% difference in euploid rates among IVF centres using donor oocyte cycles from different IVF centres, reinforcing the possible influence of the IVF laboratory on results variability.

Other factors have been proposed to induce aneuploidy and/or mosaicism as artefact of culture conditions. There are multiple factors that impact embryo development in an IVF laboratory, ranging from the egg retrieval to embryo transfer procedure, involving culture media and strategies of culture [18], air quality and purity, gamete/embryos manipulations and performance. Herein, we will emphasise on the aspects related to the PGT-A procedure that are specifically embryology laboratory related and which have not been enough explored.

Are we ready to biopsy blastocyst for PGT-A?

Concerning embryo diagnosis, prior to considering whether an embryo classified as mosaic should be transferred, one needs to understand the prevalence of real biological mosaicism in nature. True biological mosaicism has been described to be approximately 1,2% in natural conceptions and at similar prevalence in conceptions after ART treatment, at approximately 1,3% [19,20].

Differences between putative mosaicism rates across PGT-A providers, ranging from 2.9% to as high as 23.9%, raise concerns regarding the accuracy of "mosaicism" predictions [13]. This clearly suggests the presence of technical artefacts and highlights varying practices in interpreting and reporting "mosaic" results. Consequently, reporting mosaicism may primarily come at the expense of euploid diagnoses. Wiemer., *et al*. (abstract ASRM) [21] clearly demonstrated that significantly better outcome can be expected, if embryos diagnosed as "mosaic" are transferred, since most of re-biopsied, so-called mosaic embryos, are diagnosed as normal embryos [22]. Therefore, one must critically distinguish between "technical" and true "biological" mosaicism.

Concerning technical heterogenicity, one must critically question the embryo biopsy procedure. As shown by Capalbo., *et al*. [23], homogenization in training, including standardisation on the predicted amount of cells biopsied and on the type of equipment is required in order to offer reproducibility on clinical outcomes within a centre or across different IVF centres. Therefore, inter and intra-laboratory variation can impact not only on embryo viability but also on the quality of biopsy and the reported results. When there is a risk of embryo damage caused by the biopsy procedure, PGT will be limited in improving embryo selection. Without standardization of embryo biopsy techniques, one may not reliably evaluate outcomes. Apart from individual performance, the choice of methodology might as well affect outcomes and should be considered when designing RCTs. Several methods have been applied of trophectoderm (TE) biopsy with the most commonly used as follows: (a) pre-hatching embryos on day 3 to induce artificial TE herniation on day 5 or 6, facilitating TE separation for pulling during biopsy or (b) laser hatching of ZP and TE biopsy by pulling performed at the opposite side of the inner cell mass (ICM) to avoid damaging it [23]. The former (a), although seeming to be less aggressive, has been shown to result in about 10% - 20%

lower live birth rates than the latter (b) [24,25]. This might be due the earlier induction of blastocyst hatching leading to disturbances in natural blastocyst expansion, in conjunction with alteration of the natural process of trypsinization of ZP. Moreover, the pre-hatching on day 3 strategy may possibly cause herniation of the inner cell mass instead of TE cells, which will imply the need of a second laser shot on day 5, nonetheless. Although the introduction of assisted laser [26] facilitated TE biopsy and has been applied for over a decade, one must not underestimate the intensity of heat diffusion ranging between 100 and 140°C emanated by the laser shots on the neighbouring cells. The heat might be deleterious to both the TE cells that will undergo genetic analysis and the TE cells that remain with the blastocysts, and whether it may induce mosaic or chaotic changes due to cell lysis in both, remains to be elucidated. To circumvent the use of intense laser shots some utilize a "flicking" technique solely or in combination with less intensity laser, which cut cells with an abrupted movement pressing the embryo against the holding pipette [27]. Up to date, no robust reports have proven the effect of laser and/or mechanical manipulations on resulting genetic testing. Kelka., *et al*. (ASRM abstract 2017) [28] concluded that laser-assisted trophectoderm biopsy did not have an impact on genetic profiles, however, Herrero., *et al*. (ESHRE abstract 2019) [29] concluded that intense assisted laser significantly increases the risk of mosaicism compared with flicking method (17% versus 9%, $P < 0,026$).

There is a great need for a standardized embryo biopsy technique and other aspects such as timing of blastocyst vitrification and genetic analysis platforms. Moreover, whole genomic amplification (WGA) methods, in general, are prone to amplification bias, which results in low genome coverage and possible mosaicism [30]. An example is the so-called S-phase artifact, where single-cell DNA replication domains can result in copy number changes that may appear like segmental aneuploidy is an example. Although it is less likely that an entire trophectoderm biopsy would have all cells in the same phase of DNA replication, even a few cells in the replication phase may appear as a mosaic segmental aneuploidy [31]. A report by Cimadomo., *et al*. [32] exemplifies the influence of biopsy and amplification technicalities on outcomes, by demonstrating that embryos that were re-biopsied due to non informative results had a 51.9% euploid rate leading to 38.8% live birth rate.

Conclusion

We believe that each add-on technology offered to patients has its limitations and must be accompanied by appropriate and transparent discussion of the existing evidence. The controversies on the efficacy of PGT-A are, in part, due to the methodological limitations of studies which fail to consider the influence of the technical aspects on the outcomes. Although the gold standard trophectoderm (TE) biopsy procedure has yet to be established, future RCTs should take into consideration the limitations of the technicalities and apply best practice to well justify the study aim.

Conflict of Interest

There are no conflict of interest to disclosure.

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