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Letter

How PGS/PGT-A laboratories succeeded in losing all credibility

To the Editor

This letter comments on the recently published study by Romana Grati et al. (2018) and the accompanying editorial by Santiago Munné (2018). Though we are very appreciative of Romana Grati's and co-workers' efforts to improve the understanding of so-called 'mosaic' embryos after preimplantation genetic screening (PGS)/preimplantation genetic testing for aneuploidy (PGT-A), we must point out that this study, and the editorial comments by Munné accompanying it, are misleading. Both continue to build upon biologically, mathematically and logically faulty assumptions, which for far too long have been used to promote the clinical utilization of PGS/PGT-A in association with IVF.

In July of 2016, the Preimplantation Genetic Diagnosis International Society (PGDIS) created considerable confusion in issuing new guidelines for testing and reporting PGS/PGT-A (PGDIS, 2016) without concomitantly issuing explanations why those changes were made and how, without prior validation studies, such radical changes can be safely implemented. That yet another incarnation of PGS/PGT-A would be introduced without prior validation studies was especially surprising in view of earlier versions having been formally declared ineffective (Practice Committee of the American Society for Reproductive Medicine, 2008) or having been demonstrated to produce unacceptably high numbers false-positive diagnoses. The latter, of course, lead to the discarding of large numbers of perfectly normal embryos with normal pregnancy potential (Gleicher et al., 2015, 2016; Greco et al., 2015; Morales et al., 2016; Munné et al., 2017; Paulson, 2017).

Yet, this is exactly what happened with worldwide overnight adoption of the July 2016 PGDIS guidelines, which, for the first time, claimed (though in our opinion just offered up a hypothesis) that PGS/PGT-A could not only differentiate between 'normal-euploid' and 'abnormal-aneuploid' embryos but also could reliably define so-called 'mosaic' embryos as a third category, and potentially suitable for transfer (PGDIS, 2016). Since the articles by Romana Grati et al. (2018) and Munné (2018) build uncritically on these 2016 PGDIS guidelines, we demonstrate below that both communications are contradictory to fully verified biological features of human blastocyst-stage embryos.

A new concept of embryo diagnosis: the Threshold Concept

Once increasing numbers of healthy newborns were reported following transfer of so-called 'aneuploid' embryos (Gleicher et al., 2015, 2016; Greco et al., 2015; Morales et al., 2016; Munné et al., 2017), defining embryos only as either 'normal' or 'aneuploid' was no longer sustainable since there is no more convincing evidence for an embryo carrying a false-positive diagnosis than this embryo resulting in a chromosomally normal live birth. Similarly, there is no better evidence for a (much rarer) false-negative diagnosis than an embryo by PGS/PGT-A designated as 'normal-euploid' leading to an aneuploid miscarriage.

Recognizing the problem, Scott and Galliano were the first to propose a potential solution by suggesting the so-called 'threshold concept' as the next step in the continuous evolution of what then was still called PGS (Scott and Galliano, 2016). The new PGDIS guidelines, however, only a few months later, picked up on the same concept (PGDIS, 2016) and made it the new gold standard of PGS, conveniently renamed PGT-A at the same time.

Based on allegedly accurately measured aneuploid DNA loads in a single trophectoderm biopsy (TEB), embryos which up to July 2016 had uniformly been considered 'aneuploid' (and, therefore, had been uniformly disposed of) were now, suddenly, divided into two diagnostic categories, 'mosaic' and new 'aneuploid' – the latter obviously a much smaller aneuploid-category than before. A previously bimodal diagnostic scheme of 'normal-euploid' (if no aneuploid DNA was detected) and 'aneuploid' (with any amount of aneuploid DNA), now became a tri-modal scheme, with 'normal-euploid' in a single TEB defined by less than 20% aneuploid DNA, 'mosaic' by 21–80%, and 'aneuploid-abnormal' by over 80% (PGDIS, 2016). Because the new PGDIS guidelines allowed for selective transfers of 'mosaic' embryos (PGDIS, 2016), the 80% threshold now determined which embryos must be disposed of and which, potentially, could be transferred.

What was not revealed to the public and to IVF-providers, however, was that those chosen thresholds were arbitrary and lacking any

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biological and/or experimental support. The demarcation at the lower end (20% aneuploid DNA) was a purely technical one based on the minimum sensitivity level of next generation sequencing (NGS) platforms. Since other platforms were even less sensitive, the 2016 PGDIS guidelines also mandated the use of NGS platforms for PGS/PGT-A, thus formally invalidating a large variety of diagnostic platforms used routinely in laboratories up to this point (PGDIS, 2016). Just because NGS has a sensitivity level of 20% for any second cell lineage, an embryo with a hypothetical aneuploid DNA load of 19% is, therefore, considered 'normal-euploid' and indiscriminately transferrable; yet, an embryo with 21% hypothetical aneuploid DNA load is considered 'mosaic' and, therefore, is to be transferred only selectively. In other words, the sensitivity limitations of a piece of laboratory equipment now officially determine embryo fate.

The rationale behind the upper demarcation of 80% aneuploidy load for the 'mosaic' designation was even more confusing, and for the longest time remained unexplained. However, in December of 2017, Santiago Munné, in an open forum (COGI, Vienna, Austria November 30 – December 2, 2017) explained it in public for the first time in the following way: 'An average TEB usually involves 5 trophectoderm cells; 80% aneuploidy load, therefore, means that 4 out of 5 biopsied cells are aneuploid.' In other words, the new PGDIS guidelines concluded that, if 4/5 cells in a single 5-cell TEB were aneuploid, (representing 80% of total DNA in the biopsy), this was enough to declare an embryo as 'aneuploid' and, therefore, to discard it.

The absurdity of this explanation has been demonstrated with mathematical models which determined that even 100% aneuploidy in a single 6-cell TEB could not mathematically establish a high enough probability that such a finding would suggest aneuploidy for the whole trophectoderm (not even representing the whole embryo). Similarly, a mathematical model for false-negativity demonstrated that, even if all cells in a single TEB were normal-euploid, that was not enough to conclude the embryo was normal-euploid (Gleicher et al., 2017), Mathematically, a TEB with single digit cell numbers, even under unrealistically idealized mathematical circumstances of even distribution of aneuploidy throughout the trophectoderm, could not reflect the whole surface of the trophectoderm. Much larger numbers of cells in a single TEB would be required to reach this goal, and larger biopsies, of course, are not clinically feasible.

That is, however, not the only reason why Munné's explanation was so seriously flawed: no embryologist can quantitate a trophectoderm biopsy to exactly 5 cells. Moreover, as cell membranes rupture, some cellular DNA content is practically always released. What represents 80% aneuploid DNA load will, therefore, vary from biopsy to biopsy, depending on how many trophectoderm cells are included in a single biopsy specimen and whether cells are fully intact or not. In other words, Munné's explanation for the 80% threshold, between 'mosaic' and 'aneuploid-abnormal', was seriously wanting.

The new PGDIS guidelines, therefore, do not reflect validated biological thresholds, yet they determine the fate of thousands of embryos daily throughout the world. Scott and Galliano even proposed the unprecedented idea that every PGS/PGT-A laboratory determine and validate its own thresholds (Scott and Galliano, 2016), which would mean that one laboratory's threshold would not apply to the next.

Challenges to the threshold concept go even further, however. Richard Paulson explained this very well in a recently produced video (https://www.youtube.com/watch?list=PL4B448958847DA6FB&time_continue=17&v=WqT6zjRf7h8) attached to an article by Stephen S. Hall in *New York* magazine (Hall, 2017). What PGDIS guidelines describe as a 'mosaic' biopsy is really an incorrect definition of embryo mosaicism because it only reflects a single TEB of a handful of cells with, as discussed above, no practical meaning for either the complete trophectoderm or the complete embryo. Three layers of evidence support this point. As already noted, a handful of trophectoderm cells cannot reflect reliably even the total trophectoderm, let alone the inner cell mass (Capalbo and Rienzi, 2017; Orvieto et al., 2016) and the inner cell mass lineage in particular can and does, to significant a degree, self-correct downstream from blastocyst-stage (Bolton et al., 2016). The inner cell mass, moreover, is much more effective in self-correcting than trophectoderm. It should therefore not be surprising that the trophectoderm lineage – from which the placenta arises – has been known for decades to contain aneuploid cell islands even in chromosomally normal pregnancies, while the fetus – arising from the inner cell mass lineage – remains chromosomally normal.

The 32 alternating black and white panels of the soccer ball in Paulson's video demonstrate well why the location from which a random TEB is taken matters: if white panels reflect areas of only euploid cells, and black panels of only aneuploid cells, biopsies from a white panel will result in a 'normal-euploid' read of the embryo, even if all surrounding panels are black and, therefore, aneuploid. At the other extreme, if biopsies are taken from a black panel, all cells will be aneuploid, and the embryo will be reported as 'abnormal-aneuploid', even if all surrounding panels are white and, therefore, 'normal-euploid'. Since mitotic aneuploidies – in contrast to meiotic aneuploidies – are clonal, they usually occupy only small areas of the trophectoderm (i.e., the black panel will be very small), and the chance of a random TEB hitting it will be relatively low. One random TEB will, therefore, always underestimate trophectoderm mosaicism.

This explanation also points out why what PGDIS guidelines define as a 'mosaic' biopsy (i.e., presence of at least two cell lineages within one 5–7-cell TEB) is so misleading. Considering how small the chance is of hitting an aneuploid clone in a random biopsy, the chance of finding 'euploid' and 'aneuploid' cells in a single TEB is even lower, since such a finding requires that the random biopsy, by chance, be taken at an area of trophectoderm where euploid and aneuploid regions intersect. Even assuming that current diagnostic platforms do have the ability to assess accurately a second aneuploid cell lineage in a single TEB, the likelihood of having such a result is even lower than the likelihood of hitting on a small aneuploid cell clone with a single TEB.

It is therefore not surprising that the existing literature reports the prevalence of 'mosaic' TEBs in low single digits (Marin et al., 2017). Where this literature seriously errs, however, is in assuming that one TEB can realistically represent the rest of the trophectoderm and the inner cell mass. Because mitotic aneuploidies are clonal, whether the handful of trophectoderm cells in a single TEB are 'mosaic' or not, has really no representative diagnostic meaning for the total embryo.

That some proponents of PGS/PGT-A maintain that the prevalence of mosaicism in all of the trophectoderm is in the low single digits is biologically inaccurate, confusing and quite frustrating to those who are trying to make sense of current PGDIS guidelines. The correct definition of 'mosaicism' of trophectoderm is not, as PGDIS guidelines suggest, detection of one or more aneuploid cell lineages between 21% and 80% DNA load in a single TEB of 5–7 cells, but presence of two or more cell lineages *anywhere* in the trophectoderm and inner cell mass. Because of the clonal nature of mitotic aneuploidies, the more TEBs an embryo undergoes, the more 'mosaicism' will, therefore, be likely discovered.

The true prevalence of trophectoderm mosaicism in human blastocyst-stage embryos is still under debate but must be much

higher than single digits. Multiple trophectoderm biopsies from single embryos suggest at least a 50% rate (Gleicher et al., 2015, 2016). Others suggested it to be as high as 83% (Marin et al., 2017). Reproductive animal biologists consider it a virtual uniform finding at blastocyst stage (Bolton et al., 2016). A number of prepublication-stage single cell studies that we have recently become aware of also suggest that trophectoderm aneuploidy is likely to be an almost universal physiological phenomenon of day-5 human blastocyst-stage embryos.

Self-correction of embryos downstream from blastocyst stage was elegantly investigated in the mouse by Magdalena Zernicka-Goetz's laboratory in Cambridge (Bolton et al., 2016). Though initially widely dismissed by the PGS/PGT-A community (Capalbo and Rienzi, 2017), we were pleased to see that Munné in his editorial (2018) does acknowledge the increasing likelihood that such self-correction also occurs in human embryos. Assuming this to be the case, one must wonder why PGS/PGT-A at blastocyst stage would make any sense, if findings at that developmental stage do not reflect the final chromosomal fate of embryos.

The 2016 PGDIS guidelines, however, still dictate IVF practice worldwide through practically all PGS/PGT-A laboratories. Thus, a completely arbitrarily chosen and biologically non-sensical 80% threshold still determines which embryos to discard. PGDIS-recommended laboratory diagnoses and reporting as part of PGS/PGT-A, therefore, have lost all credibility.

Use of a commercial diagnostic assay under such circumstances is, likely, unprecedented in clinical laboratory medicine. It also raises serious questions about the current practice of allowing PGS/PGT-A to be offered by commercial genetic laboratories as a 'laboratory-developed' test, exempt from oversight by the Food and Drug Administration (FDA). Moreover, as a diagnostic test vested with the responsibility to determine which human embryos should be disposed of, PGS/PGT-A should definitely be subject to a review by the US Preventative Task Force, which is charged with evaluating screening tests. In other words, it appears time for federal agencies to step up oversight.

Unfortunately, the very obvious shortcomings of PGS/PGT-A outlined here define the laudable efforts of Romana Grati et al. (2018) to develop an evidence-based scoring system for prioritizing mosaic aneuploid embryos as a Quixotic experiment. If, as discussed above, the most current itineration of PGS/PGT-A does not permit a consistent and reproducible definition of 'mosaicism', how can any scoring system within this alleged 'mosaic' range demonstrate consistency and clinical validity?

Santiago Munné recently also claimed that degrees of blastocyst-stage embryo 'mosaicism' were predictive of implantation potential, with 40% DNA aneuploidy load among 'mosaic' embryos differentiating between better and poorer pregnancy chances (Munné and Wells, 2017; Munné et al., 2017). His uncritical editorial support for the manuscript by Romana Grati and colleagues is, therefore, not surprising [Munne S, 2018]. As with earlier pronouncements over the history of PGS/PGT-A, however, he is proven wrong once again: recalculating Munné's own data set (Munné et al., 2017), using ROC curves in 10% DNA load aneuploidy intervals, we found absolutely no difference in implantation/pregnancy chances at different aneuploidy loads (Kushnir et al., 2018). Of course, this negative finding should not come as a surprise, considering all of the above-outlined inadequacies of current diagnoses of 'mosaicism' by PGDIS-recommended PGS/PGT-A.

After more than 10 years of rapidly growing clinical utilization and increasing evidence that the initially promised effects of PGS/PGT-A on IVF outcomes (improved pregnancy, live birth and diminished miscarriage rates) are unachievable (Practice Committees of the American Society for Reproductive Medicine and the Society for Assisted Reproductive Technology, 2018), PGS/PGT-A remains a costly procedure in search of a clinical application. The time appears to have come to put an end to the clinical utilization of PGS/PGT-A outside of clinical trials, given that the procedure has not only failed to fulfill promises but has also has caused harm to many patients through disposal of large numbers of embryos with normal pregnancy potential. In addition, based on the mistaken assumption that they no longer produced euploid embryos, some women gave up on their own eggs prematurely and pursued egg donation unnecessarily.

The American Society for Reproductive Medicine (ASRM) and the Society for Assisted Reproductive Technology (SART) recently issued a committee opinion on PGS/PGT-A, which reached the conclusion that, 'the value of PGS/PGT-A as a screening test for IVF patients has yet to be determined' (Practice Committees of the American Society for Reproductive Medicine and the Society for Assisted Reproductive Technology, 2018). As this echoes conclusions reached ten years previously (Practice Committees of the American Society for Reproductive Medicine and the Society for Assisted Reproductive Technology, 2018) the writing appears to be on the wall: after almost 20 years of clinical utilization, and unable to find a clinical purpose for performing PGS/PGT-A, the procedure should be banned outside of clinical study frameworks.

Yet, PGS/PGT-A is still promoted in leading journals of reproductive medicine. It is our view that, to some degree, professional societies and journals should acknowledge a shared responsibility with the PGS/PGT-A laboratory community for having promoted PGS/PGT-A and that prominent members of the PGS/PGT-A laboratory community, with clear and not always disclosed commercial interests, have for years been cross-referencing and cross-reviewing each other's studies. We feel that journal editors have not only allowed that to happen but have also fostered biased reporting in the literature by promoting many of these individuals through the review process, thereby allowing them to determine what was accepted for publication and what was rejected. Concomitantly, professional societies allowed those same interested parties to dominate the profession's conferences by excluding more skeptical voices.

The history of PGS/PGT-A, therefore, offers an additional important lesson for the practice of medicine in general, in how commercial interests must not be permitted to invade peer review and medical education, to prevent them from dictating medical practice to the detriment of our patients. In this context, it is also important to note that while the clinical utilization of PGS/PGT-A has skyrocketed, live birth rates in the USA over recent years have fallen to ranges not seen since 2003–2004 (Kushnir et al., 2017). There is a lesson to be learned from this association, in that commercial interest can negatively affect outcomes in clinical medicine. Indeed, PGS/PGT-A does not appear to be the only add-on to IVF that has recently elicited such concerns in this journal (Alikani et al., 2018; Armstrong et al., 2018).

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