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REVIEW

Preimplantation genetic screening 2.0: the theory

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ABSTRACT: During the last few years a new generation of preimplantation genetic screening (PGS) has been introduced. In this paper, an overview of the different aspects of this so-called PGS 2.0 with respect to the why (what are the indications), the when (which developmental stage, i.e. which material should be studied) and the how (which molecular technique should be used) is given. With respect to the aims it is clear that PGS 2.0 can be used for a variety of indications. However, the beneficial effect of PGS 2.0 has not been proved yet in RCTs. It is clear that cleavage stage is not the optimal stage for biopsy. Almost all advocates of PGS 2.0 prefer trophectoderm biopsy. There are many new methods that allow the study of complete aneuploidy with respect to one or more of the 24 chromosomes. Because of the improved vitrification methods, selection of fresh embryos for transfer is more and more often replaced by frozen embryo transfer. The main goal of PGS has always been the improvement of IVF success. However, success is defined by different authors in many different ways. This makes it very difficult to compare the outcomes of different studies. In conclusion, the introduction of PGS 2.0 will depend on the success of the new biopsy strategies in combination with the analysis of all 24 chromosomes. It remains to be seen which approach will be the most successful and for which specific groups of patients.

Key words: preimplantation genetic screening / blastocyst biopsy / array comparative genomic hybridization / aneuploidy / chromosomal abnormalities / comprehensive chromosome screening / polar body biopsy / blastomere biopsy

Introduction

For more than 20 years preimplantation genetic screening (PGS) has been used with the aim of selecting human embryos with the highest developmental potential to improve the results obtained after assisted reproductive techniques (ART).

The overwhelming majority of human conceptions are lost, for instance, the maximum chance of a clinically recognized pregnancy occurring in a given menstrual cycle after natural conception is only ~30% (Macklon *et al.*, 2002). The mean delivery rates per aspiration for IVF, ICSI and frozen embryo replacement (per thawing) were even less: 22.4, 21.1 and 14.1%, respectively (Kupka *et al.*, 2014). Aneuploidy is probably the main cause for preimplantation growth arrest and death, as well as failed implantation, and can directly lead to implantation of an abnormal conceptus, early miscarriage, (induced) late abortion and the delivery of an affected child with a trisomy or monosomy. The majority of all embryos—be they conceived naturally or after ART—are lost preclinically and the major reason for this is aneuploidy (Macklon *et al.*, 2002).

In 1993, PGS was introduced based on the hypothesis that selection of euploid oocytes and embryos during assisted reproduction would lead to better ART outcomes (Munné *et al.*, 1993). However, after about 15 years it was demonstrated that first generation PGS was ineffective in improving IVF pregnancy rates and in reducing miscarriage rates (Mastenbroek *et al.*, 2011).

This disappointing result was at the time explained as being due to the three following causes: first, damage of the preimplantation embryo during cleavage stage following biopsy; second, incomplete and limited assessment of chromosomal status using fluorescence *in situ* hybridization (FISH); third, mosaicism of the Day-3 embryo due to postzygotic cleavage division errors (Geraedts *et al.*, 2010).

Following these insights, a new generation of PGS has been introduced. This so-called PGS 2.0, as contrasted to PGS 1.0, is characterized by polar body (PB) biopsy or trophectoderm biopsy instead of Day-3 embryo biopsy, and aneuploidy assessments of all 23 chromosome pairs instead of FISH of a limited set of chromosomes (Gleicher *et al.*, 2014).

In theory, the detection of aneuploidy in preimplantation embryos would solve many problems leading to suboptimal IVF outcomes. However, responsible innovation requires research, ideally proceeding through the steps of preclinical investigations, clinical trials and (long-term) follow-up studies (Dondorp and de Wert, 2011). The optimal design for assessing the value of PGS 2.0 would be via a prospective blind comparison of the combination of a biopsy method and a method of analysis in a given patient population. However, there are many patient groups in some of whom embryo aneuploidy may not be

the first cause of their decreased infertility. Furthermore, there are many biological (patient, oocyte and embryo) variables and different test methods with different technical limits, resulting in many different combinations and permutations that can influence the study design. In this article, we will attempt to give an overview of the variables that can theoretically influence the validity of PGS 2.0.

Aims and indications of PGS 2.0

In many patient groups the time to pregnancy matters. Therefore, the main indications proposed seem to be advanced maternal age (AMA), usually defined as maternal age above 35–38 years, repeated implantation failure (RIF) usually defined as three or more transfers of morphologically high-quality embryos without the establishment of pregnancy, recurrent miscarriage (RM) in patients with normal karyotypes (usually at least three previous consecutive miscarriages) and severe male factor infertility (usually defined as abnormal semen parameters). In all these groups of patients an increased embryo aneuploidy rate is presumed. However, the beneficial effect of PGS 2.0 has not been proved yet in a properly designed RCT in any of these groups (Gleicher *et al.*, 2014; Mastenbroek and Repping, 2014). PGS 2.0 can also be used to select the best embryo for transfer or to rank a cohort of embryos in a variety of indications including a previous sporadic genetically abnormal pregnancy, poor embryo quality and elective single embryo transfer (SET) (Coco, 2014). Furthermore, aneuploidy screening can help to assess the probability of having euploid oocytes/embryos in future ART cycles, helping patients to reach closure and help them to seek alternatives to IVF for their reproductive plans (Geraedts *et al.*, 2011; Feichtinger *et al.*, 2015).

Biopsy strategies and sample size

After PGS 1.0, it was clear that the cleavage stage is not the optimal stage for biopsy, especially since it was shown that cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not (Scott *et al.*, 2013b). An existing but not well-studied alternative is polar body (PB) biopsy. The available options are biopsy of only PBI or PBII or simultaneous or sequential biopsy of both PBs (Magli *et al.*, 2011). The main drawback of PB screening is that it only allows for the checking of maternal meiotic aneuploidies, and will not identify paternal or postzygotic mitotic error. Furthermore, often times PBs are fragmented and this quality may yield ambiguous or erroneous data.

Almost all advocates of PGS 2.0 prefer trophectoderm biopsy, because multiple cells are available after biopsy, and because this embryonic stage shows less chromosomal mosaicism (Capalbo *et al.*, 2015). However, there are concerns about long-term and transgenerational effects of culture to the blastocyst stage (Calle *et al.*, 2012). Furthermore, blastocyst stage culture is associated with increased risk of premature delivery in comparison to embryos transferred on Day 2 or 3 (Maheshwari *et al.*, 2013; Dar *et al.*, 2014).

Finally, a new source of embryonic genetic material can be obtained by blastocyst fluid aspiration (Gianaroli *et al.*, 2014). However, the reliability of this type of sample still needs to be demonstrated. The different steps are shown in Fig. 1.

Methods for comprehensive chromosome screening

Whole genome amplification (WGA), i.e. the amplification of one or two copies of the genome, can generate multiple copies in a short time and thereby result in sufficient template for comprehensive chromosome screening (Spits *et al.*, 2006). Depending on the downstream application, either multiple displacement amplification (Spits *et al.*, 2006) or library-based amplification, such as Sureplex DNA Amplification®, is used (Fiorentino *et al.*, 2014). The following methods can be used for molecular analysis of all 24 chromosomes: metaphase comparative genomic hybridization (Wells *et al.*, 1999); array comparative genomic hybridization (aCGH) (Wells *et al.*, 2008; Geraedts *et al.*, 2011); genome wide single nucleotide polymorphism analysis (Harper and Harton, 2010); PCR-based detection (Treff *et al.*, 2012) and next generation sequencing (NGS), or massive parallel sequencing (MPS) as it is currently called, using different platforms such as the MiSeq (Fiorentino *et al.*, 2014), the HiSeq platform (Wang *et al.*, 2014) or the IonTorrent platform (Kung *et al.*, 2015).

All these methods have been used to study the complete or partial aneuploidy for one or more of the 24 chromosomes. The lowest detection threshold for segmental abnormalities is different for the different methods, and so the minimal size taken into account for PGS 2.0 varies widely (Table I). Moreover, also with respect to mosaicism in multicellular samples the different methods have different detection levels. This is important since it is a matter of debate whether the aneuploidy rates in trophectoderm are a true reflection of the rates in the inner cell mass (Johnson *et al.*, 2010; Capalbo *et al.*, 2013).

Transfer policy: more complicated than expected

The number of embryos transferred is one aspect that has known an important shift over the last decade: since the introduction of the concept of elective SET (eSET) (Gerris *et al.*, 2004), it is increasingly becoming the preferred method. Furthermore, because of the improved vitrification methods, selection of fresh embryos for transfer is more and more often replaced by frozen embryo transfer (Wong *et al.*, 2014). Day 3 transfer is losing importance because of trophectoderm analysis, which requires Day 5 transfer. However, if success is measured as a cumulative rate including frozen–thawed embryo transfers, early transfer might be a better alternative. A Cochrane review on Day 2/3 versus Day 5/6 transfer in IVF showed an increased live birth rate in favour of Day 5/6 transfers. But when the meta-analysis included frozen cycles, a favourable live birth rate for Day 2/3 transfers was observed (Glujovsky *et al.*, 2012). Finally, whether to transfer or not embryos where no results were obtained after genetic analysis remains an important decision that PGS practitioners answer differently.

Success and how to measure it

The main goal of PGS has always been the improvement of IVF success rates (Gleicher *et al.*, 2014; Mastenbroek and Repping, 2014). However, success is defined by different authors in many different ways such as improved implantation rates (now largely abandoned as a success measure), decreased miscarriage rates, increased clinical

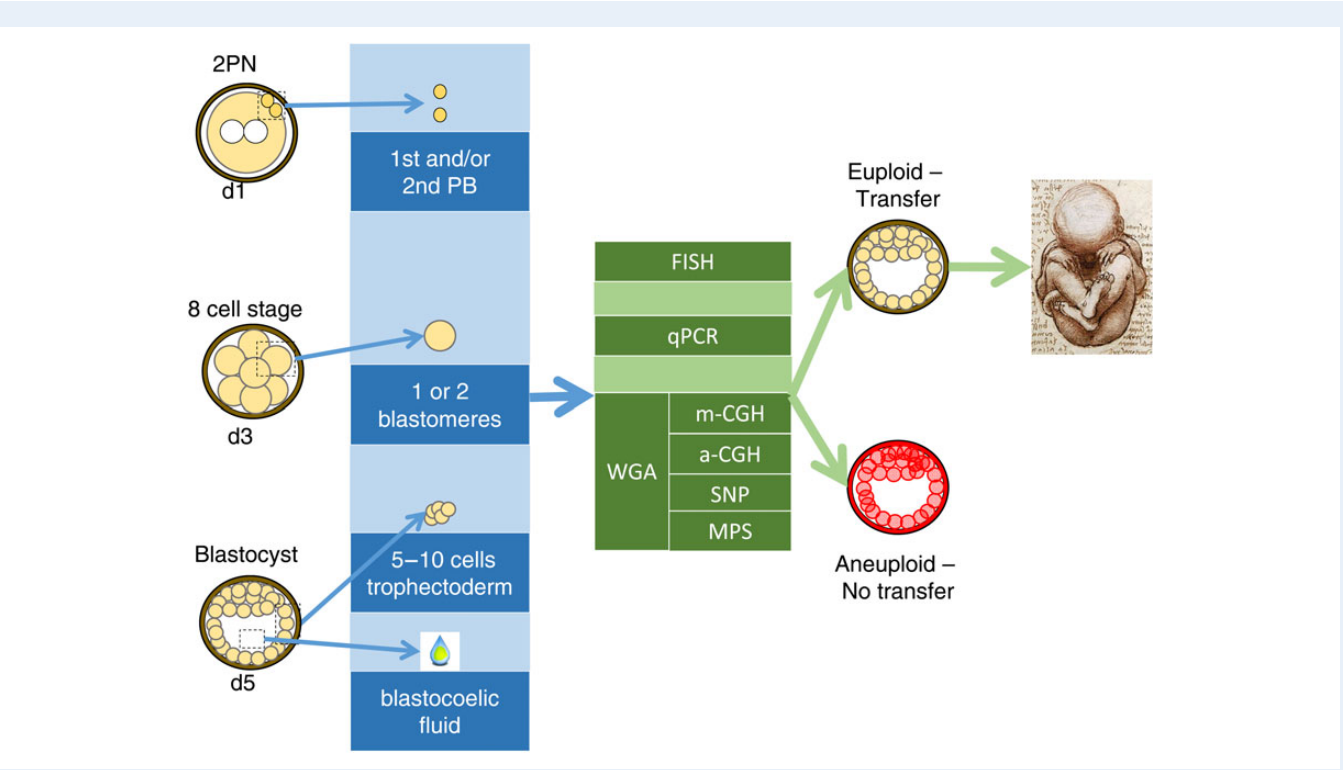

Figure 1 All aspects of preimplantation genetic screening (PGS) 1.0 and PGS 2.0. Biopsy can be performed at three different stages, either polar bodies (PB) can be biopsied from the oocyte, one or two blastomeres can be biopsied from a cleavage stage embryo, or the trophectoderm of the blastocyst can be biopsied. Finally, a small volume of the blastocoelic fluid can be aspirated. These samples can be analysed using different technologies. fluorescence *in situ* hybridization (FISH) and quantitative PCR (qPCR) do not require a pre-amplification step. Metaphase-comparative genomic hybridization (mCGH), array-CGH (aCGH), single nucleotide polymorphism arrays (SNP) and massive parallel sequencing (MPS) do require a first step of whole genome amplification (WGA). Only euploid embryos are transferred, sometimes after a period of vitrification to allow for the diagnostic procedure to take place. 2PN, two pronuclei; d1: Day 1.

Table I Detection limits for segmental aneuploidies in the human genome.

Method	Detection limits (Mb)	Reference
Metaphase cCGH	10–20	Malmgren *et al.* (2002)
BAC-array-CGH	5–10: 24Sure array (BlueGnome)	Alfarawati *et al.* (2011)
Single nucleotide polymorphism microarray	3.5: GeneChip 262 K microarray (Affymetrix)	Tan *et al.* (2013)
Quantitative PCR	Unknown	Treff *et al.* (2012)
Next generation sequencing[a]	5 (MiSeq)	Fiorentino *et al.* (2014)
	1 (HiSeq 2500)	Wang *et al.* (2014)

CGH, comparative genomic hybridization; BAC, bacterial artificial chromosome.
[a]Also called massive parallel sequencing.

pregnancy rates, improved live birth rates, prevention of the birth of chromosomally abnormal children and decreased time to pregnancy. Furthermore, success rates can be expressed in different ways: as intention-to-treat, per patient, per cycle and per transfer (fresh and frozen). This makes it very difficult to compare the outcomes of different studies and; moreover, some success measures such as implantation rates and success per transfer should not be applied. Therefore, pregnancy rates have to be calculated with cycles started rather than embryo transfers as denominator (Gleicher *et al.*, 2014).

Quo vadis, PGS 2.0?

On the basis of the variables listed above it is clear that much needs to be taken into account when evaluating the real success of PGS 2.0. In theory, it is clear that PGS 2.0 should have the potential to decrease failed implantation and miscarriage rates and therefore lead to increased pregnancy rates. PB biopsy as a variant of PGS 2.0 avoids long-term *in vitro* culture, the detrimental effect of cleavage stage biopsy and the problems related to embryo mosaicism, since mosaicism is obviously not present at the zygote stage. One argument against using PB techniques is that only the maternal aneuploidies can be detected. However, the vast majority of human aneuploidies at birth (>90%) are of maternal origin (Nicolaidis and Petersen, 1998). PB biopsy has the great advantage that more time is available to study all the chromosomes using novel molecular techniques, such as aCGH or even MPS. Furthermore, if the analysis is finished before syngamy, even restrictive laws, such as the German 'Embryonenschutzgesetz', allow this type of diagnosis (Geraedts, 2010). It is, however, very

clear that there is a trend in the PGS 2.0 literature to go to blastocyst biopsy although there is only a limited number of published RCTs (Yang *et al.*, 2012; Forman *et al.*, 2013; Scott *et al.*, 2013a). Each of these RCTs has been criticized because they were on small groups of patients, or only included patients with a transfer (Yang *et al.*, 2012), inappropriate design of the study (Forman *et al.*, 2013), or in younger patients only (Scott *et al.*, 2013a). There are no properly designed RCTs published in populations with AMA, RIF and RM (Gleicher *et al.*, 2014; Mastenbroek and Repping, 2014).

The argument has been used that vitrification and serial transfer without PGS are likely to give patients the best chance for a successful pregnancy, which means that aneuploidy screening should be avoided. The reason is that in such a freeze-all and transfer-all scenario, no selection method will ever lead to improved live birth rates, because, by definition, the live birth rate per stimulated IVF cycle can never be better than after serial transfer of all available embryos in that cycle. Furthermore, it has been argued that freezing of all embryos without PGS and serial transfer of these one by one is much more cost-effective than transfer of PGS-selected embryos.

With perfect methods for vitrification and serial transfer, selecting out and discarding embryos with $<100\%$ accuracy, as is done in PGS 2.0, could only lower the live birth rate after IVF (Mastenbroek *et al.*, 2011). However, frozen embryo transfer also carries its own costs, such as ultrasound examinations, medication, embryo thaw and culture and embryo transfer as well as indirect costs such as lost wages or cost for child care (Geraedts and Gianaroli, 2012). Embryo selection could therefore be used as an addition to vitrification and serial transfer, to rank embryos from high to low implantation potential, as the time to pregnancy may be improved by embryo selection if high quality embryos (i.e. embryos with the highest implantation potential) are transferred first. In patients of AMA, time to pregnancy is critical and its reduction through proper embryo selection is highly desirable, since without any selection it is possible to lose many costly months. Furthermore, the psychological burden of RIF and spontaneous abortion of a much wanted, clinically recognized pregnancy is severe. Transferring abnormal embryos and using natural selection mechanisms for trial and error can be seen as unethical since it is known beforehand that the majority will be abnormal—if of course embryos are correctly identified as abnormal and unable to implant. However, should all abnormal embryos be discarded? Recently, it has become known that embryos that have been diagnosed as aneuploid have given rise to healthy babies (Greco *et al.*, 2015). In this context, the major question is: what is an 'abnormal' embryo? The more ways of measuring there are, the more complicated it will get. Furthermore, the aim of testing is not only to establish a pregnancy but also to select the best embryo in order to have a healthy child. Since not all abnormalities are lethal, screening for aneuploidies can also reveal trisomy 21 conceptuses and other viable chromosome abnormalities, especially in the AMA group.

The fast technological developments in the field of genetic analysis will lead to higher throughput and lower cost. Using NGS, many samples can be run in parallel, which will make it more cost-effective than array technology. However, even when a test is shown to be effective, legal restrictions and reimbursement issues might prevent its application. Conversely, examples abound where even without demonstration of effectiveness, a test can be applied, recommended (Dahdouh *et al.*, 2015) and even reimbursed (Czech Republic).

Conclusion

The acceptance and wider introduction of PGS 2.0 will depend on the success of the new biopsy strategies in combination with the analysis of all 24 chromosomes. It remains to be seen which approach will be the most successful and for which specific groups of patients. Given the possibility of freezing and subsequently transferring all embryos obtained from a given cycle one by one, efficient PGS 2.0 would result in improving time to pregnancy by reducing the number of frozen embryo transfers needed and avoiding the related burden of implantation failures and spontaneous miscarriages. We would like to stress, however, that as long as the value of PGS 2.0 for better treatment success has not been proved by RCTs of impeccable quality, the screening should only be offered in the context of research.

In this introductory paper, we have given an overview of the different aspects with respect to the why (what are the indications), the when (which developmental stages can be sampled) and the how (which molecular technique can be used). These aspects have been translated into questions for the questionnaires for the three focus groups: geneticists—the how, embryologists—the when, and fertility experts—the why. Based on the answers given by the different experts, the companion paper will contain the practise and opinions on the future development of PGS 2.0 (Sermon *et al.*, 2016).

Authors' roles

J.G.: contributed to conception and design, acquisition of data, or analysis and interpretation of data; drafted the article and revised it critically for important intellectual content and approved the version to be published. K.S.: contributed to conception and design, acquisition of data, or analysis and interpretation of data; drafted the article and revised it critically for important intellectual content and approved the version to be published.

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Conflict of interest

None declared.

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