PREIMPLANTATION GENETIC TESTING

Clinical experience of preimplantation genetic testing

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Abstract

Thirty years of rapid technological advances in the field of genetic testing and assisted reproduction have reshaped the procedure of preimplantation genetic testing (PGT). The development of whole genome amplification and genome-wide testing tools together with the implementation of optimal hormonal stimulation protocols and more efficient cryopreservation methods have led to more accurate diagnoses and improved clinical outcomes. In addition, the shift towards embryo biopsy at day 5/6 has changed the timeline of a typical PGT clinical procedure. In this paper, we present an up-to-date overview of the different steps in PGT from patient referral to baby follow-up.

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Introduction

Preimplantation genetic testing (PGT) involves the biopsy of a single or few cells from in vitro fertilised oocytes (polar bodies) or embryos (cleavage stage or blastocyst), and testing of the biopsied cell(s) for genetic abnormalities or for HLA typing (Zegers-Hochschild et al. 2017). PGT is offered to couples with a high risk of passing on known genetic conditions, such as monogenic disorders (PGT-M) or chromosomal structural rearrangements (PGT-SR). The selective transfer of embryos unaffected for the condition under study offers the advantage of circumventing an invasive prenatal diagnosis and a possible therapeutic termination of pregnancy. PGT requires an in vitro fertilisation (IVF) treatment; this treatment with additional risks and costs may be considered as a burden for fertile couples. Couples at high genetic risk and concurrently suffering from infertility may more easily combine IVF and PGT. PGT for aneuploidies (PGT-A) with selection of euploid embryos for transfer is mainly offered to specific IVF patient groups, most commonly patients of advanced maternal age and patients with recurrent IVF failure or repeated miscarriages (not due to translocations). Here, the primary aim is to improve IVF outcomes. Although the true clinical benefit remains a topic of discussion, PGT-A is commonly performed.

The first report on children born after PGT was published by Handyside et al. in 1990, describing the use of PCR for the detection of repetitive Y-sequences for gender determination in families with X-linked diseases (Handyside et al. 1990). PGT evolved from an experimental procedure in the early nineties to a well-established clinical procedure. Numerous technical advances in the field of single cell genetic testing and biopsy methods, from targeted to genome-wide testing methods and from cleavage-stage at day 3 to blastocyst biopsy at day 5/6 respectively. As such, the classic approach of a clinical cycle with a ‘fresh embryo transfer’ has been replaced by treatments in which all embryos are cryopreserved after blastocyst biopsy while the genetic testing is pending. The genetically transferable embryos are transferred in a later, nonstimulated cycle.

The implementation of truly generic workflows with commercially available kits and protocols had two important consequences: more genetic laboratories can now provide genetic testing of single or few cells and the waiting time for couples has been substantially reduced since the customised test development steps can be omitted. Another positive evolution is that more PGT centres carry out single embryo transfers, a policy that can be linked with mechanisms of reimbursement and legislation, but also with the acknowledgement that a single embryo transfer is associated with a safer clinical outcome for any ensuing pregnancy.
As there were initially only a few specialised genetic laboratories for single cell diagnostics, PGT transport was implemented, a procedure in which embryo samples biopsied in a satellite IVF laboratory are transported to a genetics unit for testing. The provision of transport PGT is a booming service which has expanded substantially, despite the complexity of PGT and the challenges related to transport and collaborations over (long) distances.

In this review, we look back on how PGT evolved, we summarise the state-of-the-art and briefly reflect on the near future. The review has been written mainly from a European perspective and may not reflect current trends worldwide. For some aspects, we drew attention to the Belgian or the Brussels’ experience, which covers more than 25 years.

Regulation, guidelines, EQA and quality assurance

The approaches to PGT policymaking and regulation vary internationally. Many countries have a formal legislation regulating the provision of PGT (Ginoza & Isasi 2020). Some countries have a quite liberal regulation whereas other countries have more restrictive laws with designated agencies determining which centres can apply PGT (licenced centres) and for which indications. For instance, in the UK, the practice of PGT is regulated by the Human Fertilisation and Embryology Authority. In France, the Agence de la Biomédecine has a similar overseeing role, permitting PGT in case of incurable heritable disease, but disallowing comprehensive chromosome copy number testing. A minority of countries have no government regulation. The USA, for example, has no established restrictions on PGT practice and as such, PGT is also used for nonmedical reasons such as social sexing (Bayefsky 2016). A recent overview of regulatory frameworks in 43 European countries shows only two countries where PGT-M/PGT-SR is not allowed and eleven countries where PGT-A is not permitted (Calhaz-Jorge et al. 2020). The Belgian law regulating PGT was adopted in 2007, while PGT services had started many years before. The law prohibits social sexing as well as testing for nonmedical or eugenic purposes. PGT with HLA testing is exceptionally allowed for the therapeutic benefit of an existing child but the law specifies that the PGT centre should evaluate whether the couple really wishes for another child.

The costs of PGT also vary markedly between countries. For instance, in the USA, IVF and PGT treatments are usually covered by private insurances, while in France a fully free-of-charge PGT service funded by the government is available. It is clear that the differences in legislation, policies and funding have an impact on patient access to PGT. Unequal access may induce cross-border reproductive genetic care, where patients travel abroad to receive care that is not permitted or too difficult to access in their home country. Our data for PGT with HLA matching - a more controversial indication - clearly exemplify this with 56% of patients coming from abroad (De Rycke et al. 2020).

PGT guidelines and recommendations for good practice have been designed by several international societies. The PGD International Society (PGDIS) has published guidelines and position statements (PGDIS 2004, 2008, Cram et al. 2019) while the American Society for Reproductive Medicine (ASRM) reviewed PGT practice in the USA and published several opinion papers (Practice Committee of the ASRM 2008). The major mission of the PGT Consortium, founded in 1997 under the auspices of the European Society for Human Reproduction and Embryology (ESHRE) was to introduce standardisation and to promote best practice. The Consortium has recently updated and extended four sets of recommendations, covering guidance on the organisation of PGT service as well as technical guidance on embryo biopsy and genetic testing (Carvalho et al. 2020a,b, Coonen et al. 2020, Kokkali et al. 2020).

In many countries, accreditation according to national or international standards is required for both the IVF clinic and laboratory, and for the genetics clinic and laboratory. A key element in accreditation is participation in external quality assessment (EQA). The available EQA schemes for PGT were initiated by CEQAS and UKNEQAS, now merged to GENQA, in collaboration with the ESHRE PGT consortium (https://www.genqa.org/27/01/2020).

Indications

Couples opting for PGT to fulfil their child wish are referred by their physicians (gynaecologists, clinical geneticists, general practitioners or specialists in the domain of the disease they carry) or they find their way to the PGT clinic through internet searching and peer recommendation.

Currently, the most frequent indications for PGT are cystic fibrosis, myotonic dystrophy type 1, hereditary haemoglobinopathies (sickle cell anaemia and thalassemia), late-onset neurodegenerative disorders such as Huntington’s disease, hereditary cancer syndromes (e.g. BRCA1/2) and structural chromosomal anomalies (unpublished data from the ESHRE PGT consortium).

PGT-M was initially applied for severe disorders with childhood-onset and has expanded to late-onset disorders of variable severity and penetrance. Our experience with PGT for cancer predisposition syndromes shows a gradual increase with currently PGT for BRCA1 and BRCA2 genetic variants accounting for 13% of overall PGT-M cycles. A similar trend is observed from the ESHRE PGT consortium data: BRCA1/BRCA2 indications were not in the list with the 15 most frequent PGT-M indications in the overview of the first 10 years of data collection (1999–2009) while...
in the 2017 data collection, BRCA1/BRCA2 requests were the second most frequent indication (unpublished results from the ESHRE PGT consortium). For couples with a familial history of a late-onset neurodegenerative disorder, that do not wish to know their carrier status but want an unaffected child, PGT with the exclusion of at-risk embryos is offered. This is especially the case for Huntington’s disease and monogenic forms of dementia. Exclusion testing is preferred over non-disclosure testing (testing of the embryos without revealing the carrier status to the prospective parents) as the latter yields more practical and ethical issues (Shenfield et al. 2003). Some PGT-M applications have raised further ethical concerns. For instance, PGT can also be used to select embryos that are HLA compatible with an ill child who will need a bone marrow transplantation in the future. PGT with HLA matching is most often performed together with PGT for a monogenic condition (e.g. sickle cell anaemia) but it is sometimes also done for couples having a child with an acquired haematological malignancy. PGT with HLA typing is challenging as the procedure is complex and the success rates are limited, mainly because of the low genetic chance of finding an embryo genetically suitable for transfer. Few large data sets have been published, but they show that on average only 15% of diagnosed embryos are suitable for transfer (Kahraman et al. 2014, Kakourou et al. 2018, Umay Kara et al. 2019, De Rycke et al. 2020). Our own experience after two decades of PGT with HLA typing shows that it is a valuable approach in which the successful hematopoietic stem cell transplantation outcome and the positive impact on families balances the high complexity and limited delivery rate of the procedure (De Rycke et al. 2020).

PGT with sex selection is offered when a disease mainly manifests in one gender and when the search for the specific molecular defect does not have an added value (e.g. selection of female embryos in case of an AZFc deletion in the male partner). PGT with sex selection for non-medical reasons such as family balancing is prohibited in most countries.

PGT for mitochondrial (mt) DNA pathogenic variants is offered in only a few centres worldwide. It involves the selection of embryos with a mtDNA pathogenic variant load below the threshold of clinical expression. It is an ethically difficult indication as the approach reduces the risk for an affected child, rather than eliminating it and this requires case-by-case counselling (Smeets et al. 2015).

PGT referrals for double or triple conditions, monogenic and/or chromosomal aberrations are increasingly common. Not all conditions necessarily meet the ‘high risk and serious disorder’ standard, which may call for a revision of inclusion criteria and embryo transfer policy (van der Schoot et al. 2019). With the rapid advances in the field of genetic diagnostics, PGT will further expand to include non-Mendelian diseases where genetic variants in more than two genes (oligogenic) or even in tens of genes (polygenic) contribute to the disease phenotype. Instead of assessing the inheritance of a single pathogenic variant from the parent(s) to the embryo, the analysis will be based on risk scoring. A proof of concept for polygenic disease risk reduction in the context of PGT with embryo ranking based on lower risk factors was given by Treff et al. (Treff et al. 2019, 2020). Ultimately, when affordable and accurate whole-genome sequencing replaces all other methods, testing will not only assess inheritance of familial predisposition and genetic variants, but also reveal truly de novo genetic variants and molecular chromosome structure in the embryos. It is clear that the wider scope of PGT indications and technical capabilities entails an increasing complexity at the level of counselling, data interpretation and embryo transfer policies. Needless to say that this evolution should be balanced with thorough discussions and ethical reflections.

The true clinical benefit of PGT-A remains a topic of discussion. Nevertheless, PGT-A is commonly performed. The percentage of PGT-A cycles varies between 40 and 50% of overall PGT treatments, at least in the European setting of both academic and private PGT centres (unpublished data from the ESHRE PGT consortium for 2016 and 2017, 41% of all PGT cycles according to the HFEA data for 2014–2016 in the UK, Theobald et al. 2020). Worldwide, more private IVF centres are involved and the PGT-A segment is even larger with the highest use reported in the USA (Patrizio et al. 2019).

A meta-analysis of several randomised clinical trials as well as a recent paper of the Cochrane Database of systematic reviews showed no beneficial effect for PGT-A version 1.0 using cleavage stage biopsy and FISH (Mastenbroek et al. 2011, Cornelisse et al. 2020). The numerous randomised clinical trials and cohort studies on PGT-A version 2.0 versus control patients were recently reviewed (Viotti 2020). Although the studies differ in biopsy stage (polar body, cleavage-stage or TE biopsy), 24-chromosome testing method (aCGH, qPCR, NGS), embryo transfer policy (single embryo transfer, fresh or frozen) and patient group selection, the PGT-A version 2.0 data point to similar or improved live birth rates with lower miscarriage rates, fewer embryo transfers, less embryo cryopreservation and a reduced time to pregnancy (Viotti 2020). An important lesson learnt from the multicentre STAR trial is the prerequisite for optimisation and validation of laboratory procedures (Munné et al. 2019). This includes both IVF (embryo biopsy and vitrification) and genetic testing procedures. PGT-A assays are usually technically validated, for instance by doing experiments with cell mixtures of different karyotypes to model mosaicism. Clinical validation, that is, assessing the true reproductive potential of embryos interpreted as aneuploid by the PGT-A assay, is often lacking. Scott et al. published on

https://rep.bioscientifica.com
this topic following prospective, nonselection studies in which embryos were biopsied and transferred with blinded PGT-A results (SNP array-based in Scott et al. 2012 and NGS-based in Tiegts et al. 2020). Aneuploidy results, which were only disclosed after the clinical outcome became known, showed high predictive values for implantation failure. Professional organisations in both Europe and the USA have issued statements and recommendations on the responsible use of PGT-A (HFEA 2018, Practice Committees of the American Society for Reproductive Medicine in Penzias et al. 2018). This may help PGT centres in developing their own PGT-A policy and in deciding which patient groups can be included.

Genetic counselling and reproductive management

The PGT requests are evaluated by a multidisciplinary team and supplementary advice is asked to specialists when needed. The PGT requests can also be evaluated by local ethics boards. Before starting a clinical cycle, extensive genetic and reproductive counselling is required. Psychological support may be provided at any step of the procedure. The couples are informed about the different aspects of the PGT treatment, including average success rates and misdiagnosis risks.

The prospective parent(s) is/are asked to sign informed consent with regard to embryo selection by PGT. The informed consent form should include procedure-specific information, risk of false-negative diagnosis, potential risks associated with the transfer of chromosomally mosaic embryos, advice on prenatal testing following PGT, as well as issues regarding the destiny of embryos that are not genetically transferable. Specific consent is required when an exceptional transfer decision is made, for example, in cases with failed or inconclusive diagnosis or cases where the embryo transfer policy deviates from the one agreed on originally.

Genetic counselling usually starts with the verification of molecular reports from the index patient and family members because PGT cannot be offered without knowledge of the causal genetic variant. An exception can be made for families with strong evidence for an X-linked condition where PGT with sex selection can be an option in case the causal variant remains unknown. It is important to notice that for monogenic disorders, PGT will only be offered for likely pathogenic (class 4) and pathogenic (class 5) variants (Richards et al. 2015). Variants of unknown significance (VUS, class 3) are not considered for PGT since their contribution to the phenotype is not confirmed.

A three-generation pedigree is drawn to identify other potential genetic risks. Blood samples are collected from the couple for preclinical workup. Depending on the indication type, blood samples and genetic reports may also be required from relevant family members. A karyotype of both partners is equally requested and can be complemented by screening tests for carriership of common genetic variants for cystic fibrosis, spinomuscular atrophy or haemoglobinopathies. In the near future, these individual screening tests will be most likely replaced by extended carrier screening. PGT requests for more than one condition will then become less exceptional.

In case the future mother is affected by the genetic disease, a medical report should be available, stating that there is no contra-indication for a pregnancy and if necessary, specific medical investigations will be required before the start of the hormonal treatment (e.g. an MRI of the breast for female carriers of breast cancer-predisposing mutations). For these women, advice will be asked from experts in the follow-up of high-risk pregnancies.

Clinical reproductive management of the PGT patient is for many reasons more labour-intensive than conventional reproductive treatment for non-genetic indications. A major difference is the fact that up to 70% of PGT patients are not documented to have a fertility problem. Another consideration is that PGT treatment is more likely to bear the negative effects of suboptimal ovarian stimulation, fertilisation and embryo culture than conventional IVF (Verpoest et al. 2009).

The preclinical reproductive workup is similar as for patients undergoing conventional IVF. This includes ovarian reserve testing in order to determine the optimum stimulation protocol and serological testing for infectious diseases as required by local legislation. Sperm analysis is essential, most specifically for men carrying a hereditary condition potentially associated with reduced spermatogenesis. Examples are cystic fibrosis, polycystic kidney disease, myotonic dystrophy as well as Klinefelter syndrome and structural rearrangements involving the Y chromosome.

Ovarian stimulation for PGT is similar to that for conventional IVF. Ovarian stimulation protocols have not shown a significant difference in numbers of oocytes and embryos produced, although a small study on patients undergoing cleavage-stage biopsy suggested higher pregnancy rates and higher cryopreservation rates in GnRH agonist pituitary suppression stimulation programs (Verpoest et al. 2017). It is fair to state that in general, a high dose stimulation in a GnRH antagonist pituitary suppression protocol associated with GnRH agonist oocyte maturation yields less risk of ovarian hyperstimulation syndrome (OHSS) (Devroey et al. 2011) while producing an equal or higher number of oocytes than a conventional GnRH antagonist pituitary suppression protocol. This still needs to be established in studies focusing on the PGT population. A retrospective study analysing the number of oocytes required per treatment cycle in relation to the live birth rate confirmed that age and number of oocytes per treatment cycle are significant predictive outcome parameters for PGT-M (Verpoest et al. ESHRE 2019).
ICSI, embryo biopsy, transfer and cryopreservation

Intracytoplasmic sperm injection (ICSI) or the injection of a single spermatozoon directly into the ooplasm of the oocyte represented the major breakthrough for male infertility treatment (Palermo et al. 1992, Tarlatzis & Bili 2000). This fertilisation technique is specifically recommended for PGT treatment, in order to avoid paternal contamination from sperm attached to the zona pellucida as in conventional IVF (Kokkali et al. 2020). Maternal DNA contamination originating from cumulus cells can as well be reduced by careful denudation of the oocytes prior to ICSI (Wilton et al. 2009). Fertilisation rates as high as 75–80% can be obtained in the absence of a specific fertility problem. However, certain PGT indications may be associated with reduced spermatogenesis, and thus presenting with lowered fertilisation rates.

Cleavage-stage embryo biopsy has been the gold standard for many years. It implies zona opening (mechanically, chemically or by using laser energy) and blastomere removal, mainly done by aspiration, on day 3 of preimplantation development. Preclinical work from the early nineties showed that in vitro preimplantation development of biopsied human embryos is not adversely affected by the removal of one or two cells at the eight-cell stage (Hardy et al. 1990). However, later studies of our centre showed that a two-cell removal at the cleavage stage harms embryonic development and implantation potential more than the removal of one cell (Goossens et al. 2008, De Vos et al. 2009). Therefore the biopsy of one cell at the cleavage stage is recommended, provided that sufficient safeguards for a correct diagnosis are available. Polar body biopsy has been used as an alternative, albeit to a much lesser extent. This procedure is considered non-invasive for the embryo, however, its major disadvantage is that the paternal contribution to the embryo remains unanalysed. Polar body or cleavage-stage biopsy leaves sufficient time for analysis before a fresh embryo transfer. Genetically transferable embryos can be replaced on day 5 or even on day 3 in cases of polar body biopsy. If available, supernumerary genetically transferable embryos can be cryopreserved for later use.

 Blastocyst or trophectoderm (TE) biopsy is at present the most widely used technique (unpublished data from the ESHRE PGT consortium of 2016 and 2017). Laser energy is used to open the zona pellucida, either on day 3/4 or on day 5. TE cells are aspirated and excised with a laser from herniating blastocysts, or aspirated in combination with mechanical dissection from blastocysts. TE biopsy is mainly performed on day 5/6 of preimplantation development; day 7 biopsy has been reported by a minority of centres. TE biopsy provides more cells (typically five to seven) for genetic analysis and the embryonic stage is considered less sensitive to possible damage as the inner cell mass from which the fetus originates is left intact. A paired clinical trial showed that implantation rates of 50% in the nonbiopsied group were reduced to 30% in the cleavage-stage biopsy group, while for the nonbiopsied versus the blastocyst-biopsy group similar implantation rates were obtained (Scott et al. 2013). An additional benefit of TE biopsy is the lower level of chromosomal mosaicism at the blastocyst stage as compared to the cleavage stage (Viotti 2020). It needs to be underlined that TE biopsy requires an optimal blastocyst culture system. The problem of limited time for analysis in the case of a fresh embryo transfer at day 5/6 is overcome by cryopreservation (relying on efficient vitrification programs) and embryo transfer in a deferred cycle.

Whether TE biopsy will be replaced in the future by non-invasive sampling methods relying on cell-free DNA from the blastocoe1 cavity or from spent blastocyst medium requires further investigations (Leaver & Wells 2020).

Vitrification has been shown superior to slow-freezing in terms of survival rate (over 90%) for both cleavage-stage embryos and blastocysts (Loutradi et al. 2008). Clinical outcomes after transfer of euploid blastocysts in fresh IVF cycles have been proven equivalent with the outcome of vitrified blastocyst transfer cycles.

Diagnostic methods

PGT-M

Figure 1 gives an overview of past, present and future methods for PGT-M. At the start of PGT-M in the early nineties, simplex PCR amplification (two rounds with nested or hemi-nested primers) for detection of the mutation was applied (Rechitsky et al. 1999). If the mutation did not generate a difference in amplicon fragment length, digestion with suitable restriction enzymes was used for differentiation of WT and mutant alleles. Single or few cell amplification is associated with pitfalls of contamination and allele drop out (ADO), two important issues which may lead to misdiagnosis. Contamination originates from extraneous DNA or from carry-over via previous amplification reactions. ADO derives from the unequal amplification of alleles present in a heterozygous sample to the point where an allele remains undetected. By far the most important improvement to control and detect ADO and contamination was the inclusion of informative short tandem repeat (STR) markers closely linked to the region of interest. The co-amplification of STR markers with or without the mutation amplicon(s) in a multiplex PCR yields an accurate and reliable test and this haplotyping approach has been the gold standard for over two decades (Spits et al. 2006, De Rycke et al. 2017). A major limitation was that the workup (test development and validation) of a single cell multiplex PCR takes time and is quite labour intensive for the laboratory.

The implementation of a single or few cell whole genome amplification (WGA) was another major
Many WGA protocols have been published over time but none is producing a true linear representation. Computational algorithms may partially filter out the WGA pitfalls but they can not be completely eliminated and this should be taken into account during data interpretation.

WGA followed by standard PCR reactions of a multitude of STR markers flanking the region(s) of interest, is simpler and less time-consuming than direct multiplex PCR (Renwick et al. 2010). WGA followed by genome-wide methods based on SNP array or sequencing, represents a truly comprehensive approach. The current SNP array or sequencing-based approaches for PGT-M share the same principle of linkage-based testing as multiplex PCR, but the generic workflows are much more standardised and uniform, without the need for preclinical workups (Handyside et al. 2010, Masset et al. 2019). The major requirements for haplotyping are that the monogenic or the chromosomal aberration(s) is/are inherited and that relevant family samples are available. The application of generic haplotyping for balanced translocations or inversions offers the possibility to distinguish normal from balanced translocation carriers.

PGT-M is currently applicable for any (combination of) Mendelian monogenic disease, common or rare, as long as the disease causative locus has unequivocally been identified. In case of a de novo pathogenic variant(s) or when relevant family DNA samples cannot be obtained, it is necessary to include the genetic variant

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Figure 1 Overview of past, present and future methods for PGT-M. The PGT-M timeline with past, present and future sections start in the early 90s with blastomere biopsy and simplex PCR amplification for gender determination in families with X-linked diseases. Single cell multiplex PCR has been the gold standard for over two decades, alongside blastomere biopsy at day 3 and fresh embryo transfer on day 5/6. The addition of increasing numbers of flanking STR markers to the genetic variant amplicon increased the accuracy of the PCR test. Improvement of WGA methods represented a technical milestone as the tedious development of personalised multiplex PCR tests could now be circumvented. The substitution of slow-freezing by vitrification greatly improved embryo survival after cryopreservation and led to the widespread use of SET. It also alleviated the need for a fresh transfer, removing analysis time constraints. By shifting to TE biopsy more cells can be biopsied, yielding more DNA and hence improving test accuracy. Genome-wide methods such as SNP array and sequencing were developed for PGT-M. These truly generic methods allow diagnosis for virtually any known monogenic disorder in a single workflow. In addition PGT-M can be combined with PGT-SR and PGT-A. As a result, TE biopsy at day 5/6, combined with WGA, a genome-wide analysis and a freeze-all strategy is currently increasingly used. In the future, whole-genome sequencing will replace all other methods. We expect that its use for detection of de novo variants (de novo in the embryo) and diagnosis of severe oligo/polygenic disorders will render data interpretation and policy-making more complex. Ideally, TE biopsy will be replaced by a reliable non-invasive sampling method in the future. Adapted from De Rycke and Berckmoes (2020). PCR + FA: PCR followed by fragment length analysis. Analysis of STR markers can be combined with detection of the pathogenic variant using a variety of technologies (fragment length analysis, minisequencing, allele-specific amplification, etc.). WGA, whole genome amplification. WGA products are used as DNA source for downstream applications such as PCR + FA, SNP array or massive parallel sequencing. STR, short tandem repeat marker; SNP, single nucleotide polymorphism marker; SET, single embryo transfer; TE d5/6, trophectoderm biopsy at day 5 or 6 after fertilisation; seq, sequencing.
detection in the test strategy and to determine high-risk and low-risk haplotypes during clinical cycle(s). At our centre, currently up to 14% of PGT-M requests fit within this category. Deletion or duplication variants of a few nucleotides can be detected directly by fragment length difference. For single nucleotide substitutions, different strategies of allele discrimination have been developed, for example, minisequencing (Bermudez et al. 2003). The direct detection of complex and/or larger gene rearrangements has so far been difficult since the exact breakpoints of the rearrangement are frequently unknown. High-resolution characterisation of breakpoints located outside highly repetitive regions is now achievable with long-read nanopore sequencing (Chow et al. 2020).

As generic haplotyping approaches generate both genotype and chromosome copy number info from the same raw data set, it is feasible to routinely add PGT-A to PGT-M/SR cycles. Concurrent PGT-M/SR and PGT-A is increasingly regarded as an acceptable option, since it does not entail additional procedures or higher risks for the couple or future child. So far, few well-developed algorithms providing an all-in-one solution for data interpretation have been published. Some PGT centres rely on in-house developed extended or novel algorithms to overcome this limitation (e.g. Haplarithmisis (Zamani Esteki et al. 2015)). These algorithms not only allow haplotype and chromosome copy number detection, they can as well identify the nature (meiotic or mitotic) and the parental origin of the chromosomal aneuploidy.

It is clear that the implementation of concurrent PGT-M/SR and PGT-A offers further possibilities but it also generates more complex genetic information which we currently do not fully understand. The innate embryo chromosomal mosaicism for instance entails many ethical discussions and challenges for genetic counselling because we lack knowledge to support data interpretation. The definition of chromosomal mosaicism is the co-presence of cells with two (or more) different chromosomal constitutions. In the context of PGT-A, the most relevant type is the mix of euploid and aneuploid cells (sometimes referred to as diploid-aneuploid mosaics (Viotti 2020), hereafter simply referred to as mosaic). Professional organisations and working groups have issued position statements on the transfer of mosaic embryos (for instance PGDIS 2019, Gleicher et al. 2020 and COGEN) offering good practice recommendations and guidance. Our internal guidelines for mosaic embryo transfer are based on available literature (amongst others Greco et al. 2015, Dimitriadou et al. 2017, Fragouli et al. 2017, Munné et al. 2017, Grati et al. 2018, Kushnir et al. 2018, Spinella et al. 2018, Popovic et al. 2019, Victor et al. 2019, Zhang et al. 2019, Zore et al. 2019). Our centre relies on SNP array with an in-house developed algorithm for concurrent PGT-M and PGT-A. Cut off values for mosaicism (>25 and <75%) were established following validation with euploid/aneuploid cell mixtures. Embryos are classified in three groups based on their risk to yield live born affected children. Low-risk euploid embryos are prioritised above low-risk mosaic embryos whereas high-risk mosaic embryos are considered not genetically transferable. The latter high-risk group includes embryos showing either aneuploidy (whole chromosome or segmental) in all cells or embryos showing mosaic disomy/trisomy for chr 8, 9, 13, 16, 18, 21 and X or mosaic monosomy/disomy X. All other whole chromosome mosaic embryos are within the low-risk mosaic group. Segmental mosaic embryos are considered case by case. Patients receive appropriate counselling and follow up. These internal guidelines also apply for PGT-SR and PGT-A, with the exception that embryos showing mosaic aneuploidy for a translocation chromosome are classified in the high-risk group.

Data from the last several years show that the transfer of mosaic embryos may yield healthy babies (compiled data analysis in Viotti 2019). However, implantation rates are lower and miscarriage rates are increased in comparison with euploid embryo transfer (Greco et al. 2015, Fragouli et al. 2017, Kushnir et al. 2018, Victor et al. 2019, Zhang et al. 2019, Zore et al. 2019, Munné et al. 2020). It is fair to state that the exact risk of transferring a chromosomally mosaic embryo cannot be precisely determined. First, the observed degree of mosaicism may not be representative of the inner cell mass. Secondly, very large datasets on the outcome of transferred mosaic embryos are still missing. In this knowledge gap, PGT clinics and patients struggle to find the balance between the highest chance of obtaining a pregnancy and the lowest risk for an abnormal foetus. The Practice Committee and Genetic Counseling Professional Group (GCPG) of the American Society for Reproductive Medicine published guidance (2020) on how to counsel patients on these complex issues.

**PGT-SR and PGT-A**

Figure 2 gives an overview of past, present and future methods for PGT-A/SR. Familial reciprocal and Robertsonian translocations constitute the most common indications for PGT-SR. Other indications for PGT-SR are deletions, duplications, inversions, insertions and more complex chromosomal rearrangements.

To date, the most applied methods for PGT-SR include targeted fluorescence in situ hybridisation (FISH) or genome-wide WGA followed by array-based comparative genomic hybridisation (aCGH), low coverage sequencing or SNP array.

FISH was the first method to be applied for PGT-SR and has been the gold standard for over two decades. It necessitates a preclinical workup with optimisation of probe mixtures for each individual rearrangement, in order to maximise and guarantee the test performance. Single cell FISH is technically difficult: the initial step of
cell fixation is critical and requires skill and experience. Furthermore, the technique is labour intensive and interpretation may be subjective due to signal overlap, split signals, high background signals or low signal intensity. As a result, FISH is gradually replaced in many laboratories by genome-wide methods. Currently, in many PGT centres FISH is only applied for rearrangements for which no other suitable methods are available.

The first widely used generic genome-wide technique for PGT-SR was aCGH. With this technique, unbalanced segments larger than 5–10Mb are reliably detected and therefore aCGH can be applied for the vast majority of translocations. The abrupt discontinuation of the most commonly used platforms (24Sure and 24Sure+, Illumina) urged shifting to low coverage sequencing in many labs.

Low coverage sequencing has a resolution for the detection of unbalanced chromosome segments of 5Mb or lower and so it is performing equally or better compared to aCGH. As the detected copy number change is a better reflection of the true copy number change the detection of mosaicism is more reliable. There are both commercially available platforms (Veriseq, Vitrolife) and in house developed platforms (Deleye et al. 2018; unpublished results).

The techniques of aCGH and low coverage sequencing share similar strengths and limitations. Strong assets are that all 24 chromosomes can be assessed and that there is no need for a preclinical workup. Weaker points are that normal and balanced segregations of structural rearrangements cannot be distinguished and that some types of abnormalities such as uniparental disomy, haploidy or triploidy cannot be identified, as only relative chromosome copy number changes can be detected.

In recent years also SNP haplotyping methods such as SNP array-based Karyomapping (Vitrolife) and the high
coverage reduced representation sequencing-based SiCHILD/haplarithmisis/OnePGT (Agilent) have been applied for PGT-SR (Handyside et al. 2010, Zamani Esteki et al. 2015, Masset et al. 2019). These methods have the advantage that also small deletions, duplications and reciprocal translocations with small exchanged segments can be reliably diagnosed using haplotype information. Normal and balanced segregations can be distinguished. Detection of aneuploidy is also possible (OnePGT, Karyomapping). The flipside of the coin is the requirement of a preclinical workup and the availability of a DNA sample from a family member with the same balanced structural rearrangement as a reference. Do note that for small segment (<5 Mb) rearrangements, diagnosis primarily relies on flanking haplotype information and therefore the genomic context and structure of the rearrangement needs to be known. Especially in case of observed copy number gains, duplications need to be discriminated from (rare) unbalanced insertions.

With the development of new techniques for breakpoint detection at basepair level using long-read sequencing (Chow et al. 2020), it becomes possible to design amplicons bridging the rearrangement breakpoints. This in turn allows segregation analysis between breakpoint and SNP haplotype in embryos. In this way, PGT can also be offered to patients with chromosomal rearrangements involving segments smaller than 5Mb, relying on haplotyping without a reference. The FISH-free PGT-SR clinic is within reach.

PGT-A, formerly known as preimplantation genetic screening (PGS) was first applied with FISH for a limited number of chromosomes. Nowadays the genome-wide methods described above are applied on TE biopsies. These methods allow reliable aneuploidy detection but the frequent detection of chromosomal mosaicism poses a new challenge, as discussed in the previous section.

**Clinical outcome**

This section includes results from our centre; sharing this data may help to counsel patients in terms of expectations. Considering the many technical improvements from both the genetic testing and the IVF laboratory side, only clinical results from the last years are presented here. Our centre performs about 600–700 cycles per year, mainly PGT-M and PGT-SR. PGT-A cycles represent about 10–15% of our PGT activity. The mean female age is 33 years. These patients present with an average of 11–13 cumulus oocyte complexes. ICSI fertilisation is 75–80%. In cases of cleavage-stage biopsy, about 80% of the fertilised oocytes can be biopsied on day 3. Transfer rate for PGT patients is around 70% and cancellation is mostly related to the fact that no genetically transferable embryos are available or to insufficient blastocyst quality. Positive hCG rates are up to 40% per started cycle or 55% per embryo transfer (2013–2016). Clinical pregnancy rates with foetal heartbeat reach 42% per embryo transfer.

TE biopsy was introduced in our centre in 2014 and today it corresponds to 60% of all biopsy cycles. On average 40–50% of fertilised oocytes can be biopsied on day 5 or day 6. About 14% of cycles scheduled for trophectoderm biopsy are cancelled as embryos do not reach the blastocyst stage or embryo quality is insufficient for biopsy. In all TE biopsy cycles, biopsied blastocysts are vitrified awaiting the genetic diagnosis. If genetically transferable, they are replaced in an unstimulated frozen embryo transfer cycle. Positive hCG rates per embryo transfer are 55–60% (2016–2019). Clinical pregnancy rates with foetal heartbeat reach 50% per frozen embryo transfer (2016–2018). One of the various factors which may influence the final outcome is the number of embryos suitable for transfer. Cycles for autosomal dominant conditions have a markedly lower clinical pregnancy rate per cycle (37%) compared to autosomal recessive conditions (47%) in our PGT-M cohort (unpublished data from 2016 to 2018). This is in accordance with the lower theoretical chance for genetically transferable embryos in case of autosomal dominant disorders. The lowest number of embryos suitable for transfer (16%) and associated outcome (clinical pregnancy rate of 32% per transfer or 16 % per started cycle) are observed in our subset of cycles with HLA matching (De Rycke et al. 2020). Our results are in line with those of a Canadian PGT centre reporting ongoing pregnancies rates of 49% for PGT-M and 43% for PGT-SR (Butler et al. 2019).

It is obviously difficult to compare clinical outcomes between individual centres because of varying factors such as maternal age, indication type or differences in genetic testing and IVF procedures. Alternatively, comparisons of clinical outcome with means reported by consortia can be used. The publication of large data collections like the ESHRE PGT consortium has been lagging, hindering proper benchmarking. The ESHRE EIM consortium has published summary data on PGT for 2014 (De Geyter et al. 2018). This data set, which has been collected from 22 countries, shows a pregnancy rate of 42.5% per fresh embryo transfer cycle and a rate of 41.8% per frozen embryo transfer, similar to the outcome results mentioned above.

Apart from the number of transferable embryos, other important factors influencing the clinical outcome are the number of oocytes and the number of blastocysts for biopsy, as reported by Ben-Nagi et al. for a cohort of PGT-M/PGT-SR cycles with concurrent PGT-A (Ben-Nagi et al. 2019).

**Children follow-up**

At our centre, we have developed a standardised follow-up program. Before starting PGT treatment, couples give their written informed consent to a prospective clinical follow-up of cycles, pregnancy and children. Written
data on pregnancy and delivery are obtained from the attending obstetrician and pediatrician when necessary. For families from abroad, information is retrieved from detailed questionnaires filled out by the parents, gynaecologists and/or pediatricians. In case of missing information, parents will be interviewed by letter or/and by phone. Belgian families are invited to our centre for a thorough medical examination of their child at the age of 2 months and at 2 years. The examination consists of two parts. The first part involves a structured interview about the birth parameters, the child’s health and development. The second part is a clinical evaluation with the collection of biometric data (height, weight and head circumference), evaluation of neurological and psychomotor development and screening for major and minor anomalies following previously published criteria (Van Steirteghem et al. 2002, Bonduelle et al. 2003).

Follow-up data on children conceived after PGT are still limited. It is hypothesised that assisted reproductive technologies (ART), especially the more invasive techniques like ICSI or embryo biopsy, would increase the risk for birth defects. Studies showed that IVF and ICSI are associated with a small but statistically significant increase in congenital anomalies at birth, compared with the general population (Davies et al. 2012, Pandey et al. 2012). No difference was observed between ICSI and IVF (Bonduelle et al. 2003, 2005, Zhu et al. 2019).

For PGT, clinical studies did not report a higher rate of major congenital malformations when compared to IVF/ICSI children (Banerjee et al. 2008, Desmyttere et al. 2009, 2012, Liebaers et al. 2010, Bay et al. 2016, Heijligers et al. 2018). Many of these studies contain data on pregnancies and children born after preimplantation aneuploidy screening (PGT-A) and thus include couples with a history of infertility or advanced maternal age. It is well known that pregnancies following fertility treatment in infertile couples carry an increased incidence of preterm delivery and lower birthweight.

The study from our centre compared neonatal data of 995 live-born children after PGT with the outcome data of 1507 live-born children after ICSI and showed that embryo biopsy at the cleavage stage did not add a significant risk to the overall medical condition of newborn children nor did it change the risk for major malformations (Desmyttere et al. 2012). These results were in line with studies carried out in other centres using embryo biopsy at the cleavage stage (Bay et al. 2016, Heijligers et al. 2018).

The recent retrospective study in which a cohort of 1,721 children born after PGT with blastocyst biopsy and cryopreservation was compared with an IVF/ICSI control group, showed no significant difference in neonatal outcome, indicating that neither embryo biopsy at blastocyst stage nor cryopreservation added further risk to the health of PGT children (He et al. 2019).

The follow-up of PGT children at young age has been studied in smaller cohorts. Developmental neurological and cognitive assessment and follow-up on psychomotor and social functioning showed that PGT pre-schoolers were comparable with controls born after ICSI or after spontaneous conception (Winter et al. 2014, 2015, Sacks et al. 2016, Heijligers et al. 2019). Another study on body composition and blood pressure showed no adverse outcomes for 6-year-old children born after PGT (with day 3 embryo biopsy followed by blastocyst transfer) compared to children born after ICSI without embryo biopsy (Belva et al. 2018). In summary, the follow-up results have so far been reassuring but further monitoring of the safety of PGT and the long-term health of the children remains necessary.

Conclusions and future perspectives

Major advancements have been introduced in the area of preimplantation genetic testing and assisted reproduction over the years, making PGT a well-established, accurate and safe clinical procedure. The implementation of genome-wide methods has allowed more standardisation and uniformity for the genetic laboratories. However, additional genetic findings other than the requested genetic condition, such as chromosomal mosaicism in embryos tested for a monogenic disorder, have posed new dilemmas for genetic counselling and embryo transfer policymaking. As the cost of sequencing continues to decline, PGT moves technically towards a sequencing-based, all-in-one solution for PGT-M, PGT-SR and PGT-A. As there is more awareness with patients about the risks of transmitting genetic disorders and since the number of diseases with identifiable genetic cause(s) continues to rise, the total of treatments as well as the list of indications for PGT is likely to expand. Whether in the future the scope of PGT indications will broaden from diagnosis of monogenic disorders to also predicting the risk of polygenic disorders represents an emerging ethical challenge for PGT practice. It is clear that the rapid technological advances should be balanced with ethical reflection and thorough discussions.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Clinical experience of PGT


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