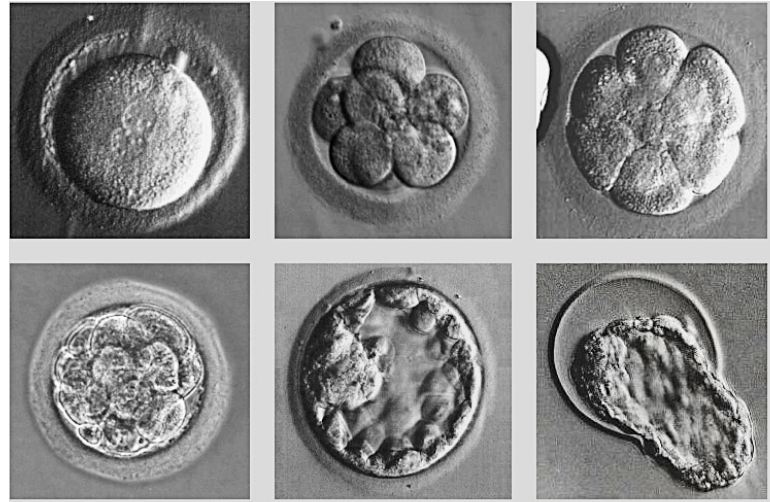




Preimplantation genetic testing for monogenic disorders in transport service: comparison of a targeted testing *versus* a genome-wide testing approach

Beatriz Dias

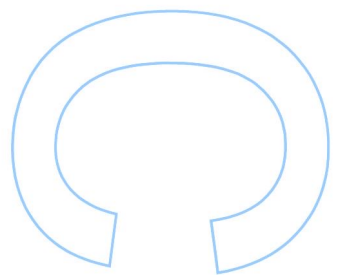
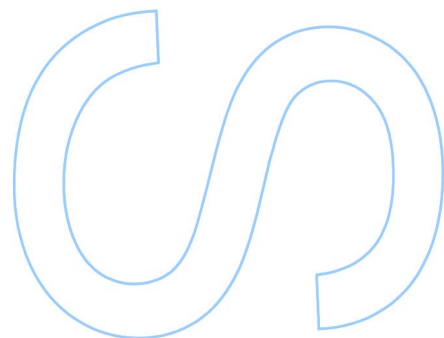
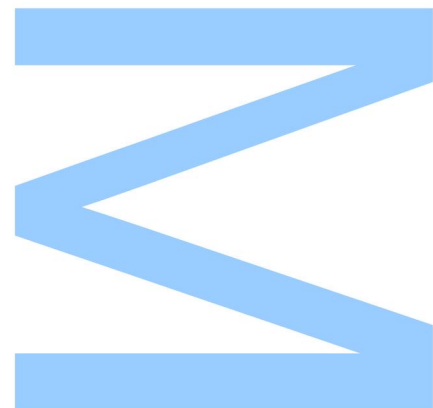
Master Program in Biochemistry
2021

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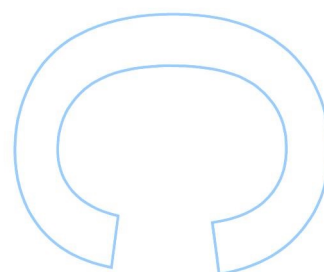
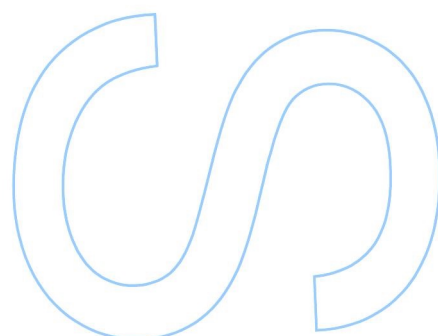
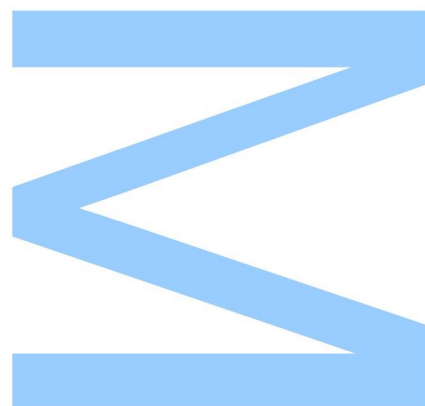




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Master Program in Biochemistry

Chemistry and Biochemistry Department

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Abstract

Preimplantation Genetic Testing (PGT) involves the biopsy of a single or few cells from *in vitro* fertilized embryos and testing of the biopsied cell(s) for genetic abnormalities or for HLA typing. It can be performed for aneuploidy detection (PGT-A), for monogenic disorders (PGT-M) and for chromosomal structural rearrangements (PGT-SR). PGT can be executed *in-house*, meaning that the IVF center and the genetic diagnosis center are located in the same institution, or in transport PGT, meaning that fertilization, embryo culture and embryo biopsy are performed in a satellite IVF center and the biopsied cell samples are transported to a genetic diagnosis center. After the diagnosis, embryo transfer is also carried out in the satellite IVF center, as well as the follow up of the pregnancies and the resulting children.

Transport PGT-M between the Genetics Department of the Faculty of Medicine of the University of Porto and the Centre for Medical Genetics of UZ Brussel started in 2006. Since then, transport PGT-M preclinical workups and cycles were carried out for almost 300 couples. For a long time, a day 3 biopsy followed by a single cell Multiplex-PCR (targeted testing) was carried out, but recently, a day 5/6 biopsy followed by whole genome amplification and SNP array (SNPa) (genome-wide testing) was implemented.

The aim of the project is to compare, for transport PGT-M cycles, day 3 biopsy/targeted testing with day 5/6 biopsy/genome-wide testing method for specific parameters (number of embryos tested, number of embryos genetically suitable for transfer, efficiency of the genetic test, contamination and allele dropout rates and clinical outcome). To assess possible consequences of the transport of the biopsied cell samples, transport cycles were also compared with *in-house* cycles, for the same parameters.

Our data clearly shows numerous advantages of day 5/6 biopsy followed by genome-wide technology - preclinical workups tend to be ready faster, the diagnosis efficiency is higher and only euploid embryos are transferred. A superior implantation, clinical pregnancy, and live birth delivery rates are observed. There seems to be no increased risk in preterm delivery and low birth weight. Therefore, a day 5/6 biopsy followed by a genome-wide technology for diagnosis, such as SNPa, seems to be a superior strategy considering PGT treatment.

Keywords: Preimplantation Genetic Testing, embryo biopsy, targeted approach, genome-wide approach, implantation, pregnancy.

Resumo

O teste genético de pré-implantação (do inglês, *Preimplantation Genetic Testing*, PGT) envolve a biópsia de uma ou algumas células de embriões fertilizados *in vitro* e o teste da(s) célula(s) biopsiada(s) para anomalias genéticas ou para tipagem HLA. Pode ser realizado para detecção de aneuploidias (PGT-A), doenças monogénicas (PGT-M) e rearranjos cromossómicos estruturais (PGT-SR). O PGT pode ser executado internamente, o que significa que o centro de FIV e o centro de diagnóstico genético estão localizados na mesma instituição, ou em transporte, o que significa que a fertilização, a cultura dos embriões e a biópsia embrionária são realizados num centro de FIV satélite e as células biopsiadas são transportadas para um centro de diagnóstico genético. Após o diagnóstico, no centro de FIV satélite é realizada a transferência embrionária, bem como o acompanhamento das gestações e das crianças resultantes.

Desde 2006, existe uma colaboração de PGT-M de transporte entre o Serviço de Genética da Faculdade de Medicina da Universidade do Porto e o Centre for Medical Genetics of UZ Brussel. Desde então, foram efetuados exames pré-clínicos e ciclos de PGT-M de transporte para quase 300 casais. Durante muito tempo, a biópsia do dia 3 seguida por PCR Multiplex de uma célula única (teste direcionado) foi realizada, mas recentemente, a biópsia do dia 5/6 seguida pela amplificação do genoma completo e SNP Array (SNPa) (teste de todo o genoma) foi implementada.

O objetivo deste projeto é comparar, para os ciclos de PGT-M de transporte, a biópsia do dia 3 / teste direcionado com a biópsia do dia 5/6 / teste de todo o genoma para parâmetros específicos (número de embriões testados, número de embriões geneticamente transferíveis, eficiência do teste genético, taxas de contaminação e *allele dropout*, resultados clínicos, ...). Para avaliar possíveis consequências do transporte das células biopsiadas, os ciclos de transporte foram comparados com ciclos internos.

Estes dados mostram claramente inúmeras vantagens da biópsia do dia 5/6 seguida por tecnologia de todo o genoma – os testes pré-clínicos são mais rápidos, verifica-se uma maior eficiência no diagnóstico e apenas embriões euploides são transferidos. São observadas taxas de implantação, gravidez clínica e nascimento superiores. Parece não haver risco aumentado de parto prematuro e baixo peso à nascença. Assim, a biópsia de dia 5/6 seguida por uma tecnologia de análise de todo o genoma para o diagnóstico, como SNPa, parece ser uma estratégia superior para o PGT.

Palavras-chave: Teste Genético Pré-implantação, biópsia embrionária, abordagem direcionada, abordagem de todo o genoma, implantação, gravidez.

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List of abbreviations

AA	Aminoacids
aCGH	Microarray-based Comparative Genomic Hybridization
AD	Autosomal dominant
ADO	Allele <i>dropout</i>
ALB	Alkaline lysis buffer
AMA	Advanced maternal age
AR	Autosomal recessive
ART	Assisted reproduction technologies
BAF	B allele frequency
bHCG	beta Human Chorionic Gonadotrophin
BMI	Body mass index
CE	Capillary electrophoresis
CF	Cystic Fibrosis
CNPMA	National Council for Medical Assisted Reproductive Technologies
CNVs	Copy Number Variations
COH	Controlled ovarian hyperstimulation
CPR	Clinical pregnancy rate
CVS	Chorion villus sampling
ddNTP	Dideoxy nucleotide triphosphate
DMD	Duchenne Muscular Dystrophy
DNA	Desoxyribonucleic acid
DTT	1,4-dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESHRE	European Society of Human Reproduction and Embryology
FISH	Fluorescent <i>in situ</i> hybridization
HLA	Human Leukocyte Antigen
HD	Huntington disease
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IVF	<i>In vitro</i> fertilization
KLI	Clinical protocol
LBDR	Live birth delivery rate

LBW	Low birth weight
MDA	Multiple displacement amplification
NGS	Next-generation sequencing
OHSS	Ovarian hyperstimulation syndrome
OMIM	Online Mendelian Inheritance in Man
PB	Polar body
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGD	Preimplantation genetic diagnosis
PGS	Preimplantation genetic screening
PGT	Preimplantation genetic testing
PGT-A	Preimplantation genetic testing for aneuploidies
PGT-M	Preimplantation genetic testing for monogenic disorders
PGT-SR	Preimplantation genetic testing for structural rearrangements
PND	Pre-natal diagnosis
QA	Quality assurance
QC	Quality control
RIF	Recurrent implantation failure
RM	Recurrent miscarriage
SMF	Severe male factor
SNPa	Single nucleotide polymorphism microarray
SNP	Single nucleotide polymorphism
STRs	Short-tandem repeats
TAT	Turnaround Time
TE	Trophectoderm cells
VLBW	Very low birth weight
WGA	Whole genome amplification
X-linked D	X-linked Dominant
X-linked R	X-linked Recessive
ZP	Zona Pellucida

1. Introduction

1.1 Historical background

“The double helix is indeed a remarkable molecule. Modern man is perhaps 50,000 years old, civilization has existed for scarcely 10,000 years and the United States for only just over 200 years; but DNA and RNA have been around for at least several billion years.”

Francis Crick (1916–2004)

Genetics is a branch of Biology that dates from 1866, when Gregor Mendel, also known as the “father of genetics” suggested for the first time that some characteristics could be transmitted to the next generation [1]. In 1866, Haeckel proposed that factors enclosed within the nucleus were responsible for hereditary traits’ transmission [2], but it was only in 1869 that Swiss chemist Friedrich Miescher isolated and identified the molecule responsible for this transmission, in the cell nucleus, today known as DNA (desoxyribonucleic acid) [3]. Since then, multiple scientists, from different disciplines, largely contributed to the understanding of the constitution of DNA, its function, and its mechanisms.

Walter Sutton and Theodor Boveri were two other very important scientists in the field of genetics, proposing the Chromosomal Theory of Inheritance, in 1902 [4]. Thanks to the continuous technical development of important instrumentation in the field, such as the microscope, Sutton and Boveri were able to observe chromosome behavior during cell division, which allowed them to witness chromosome segregation during anaphase. In the same year, Archibald Garrod noticed that alkaptonuria was inherited according to Mendelian rules [5]. He was also the first to use the expression “inborn errors of metabolism”, but it was only in 1909 that the word “gene” was firstly used by Wilhelm Johannsen, as well as the terms “phenotype” and “genotype” [6]. In his book, “Introduction to the elements of an exact theory of heredity”, Johannsen defines gene as “something that is found in the gametes and participates in determining specific properties in a developing organism” [7].

All of this research was then deepened by Thomas Hunt Morgan, thanks to his experiments with *Drosophila melanogaster* [8], using both the Chromosomal Theory of Heredity and Mendelian Genetics [4]. His work led to a publication, in 1915, that became one of the most important books in genetics’ history – “The Mechanism of Mendelian Heredity” [9]. As a consequence, Morgan was awarded the Nobel Prize in Physiology or Medicine in 1933, “for his discoveries concerning the role played by the chromosomes in heredity” [10]. Following his

work, Morgan' student, Alfred Henry Sturtevant manage to draw the first chromosomal linkage map for the genes located in the X chromosome of fruit flies [11].

Between 1951 and 1952, Alfred Hershey and Martha Chase conducted a series of experiments that led to the conclusion that genes are made of DNA [12]. One year later, two scientists from the University of Cambridge, James Watson and Francis Crick, unraveled the tree-dimensional structure of the DNA molecule, demonstrating how DNA functions as genetic material [13]. "For their discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material", Watson and Crick were awarded the Nobel Prize in Physiology or Medicine, in 1962 [14], along with Maurice Wilkins, who worked together with Rosalind Franklin in the discovery of DNA's tridimensional structure, using X-ray crystallography [15]. Finally, in 1955, Joe Hin Tijo defines 46 as the exact number of chromosomes in a human cell [16].

The next years were marked with numerous findings, from enzymes responsible for DNA replication, the semi-conservative process of replication, the discovery of RNA (ribonucleic acid), up to the discovery of the genetic code. Various methodologies were found to sequence DNA. In 1983, Huntington Disease (HD) was the first mapped genetic disorder. Gusella and his colleagues found a genetic marker linked to HD on chromosome 4, but the gene itself was not finally isolated until 1993 [17]. In the same year, Kary Mullis invented the Polymerase Chain Reaction (PCR), which granted him the Nobel Prize in Chemistry in 1993 [18].

In 1988, The United States of America National Research Council suggested a program to map the entire human genome, today known as the Human Genome Project. This project had its official start in 1990. In 1999, an international team of researchers reached a major milestone when they unraveled for the first time the full genetic code of chromosome 22 [19]. Chromosome 22 was the first of the 23 chromosomes to be deciphered, mainly due to its small size and its association with several diseases. Five years later, the Human Genome Project was concluded [20], estimating that the human genome has 20.000 to 25.000 genes, a smaller number than expected.

1.2 Genes and Diseases

A gene is a specific length of DNA that encodes for a specific order of amino acids, which generates a protein. Each gene has two alternative forms within an individual, called alleles, each one inherited from each parent. Those alleles may be similar to each other, and therefore the person is considered homozygous for that gene, or be different, thus the person is considered heterozygous for that gene [21]. However, the genetic material can have one or more alterations, commonly known as mutations, that can be transmitted, or not, to the offspring. These mutations can occur all over the genome, gaining a major importance when

affecting genes. If the mutation is present in the gamete cells, it can be passed through generations [22]. A mutation is then a permanent change in the nucleotide sequence, which can be distinguished from a polymorphism, that is a variant in the genome with a frequency above 1%. In 2015, due to the frequent confusion of the terms “mutation” and “polymorphism”, it was proposed that both terms should be replaced by the expression “variant” [23].

We can distinguish different kinds of variants, based on the length of the affected region. Roughly, we can subdivide these variants in three classes, summarized in Table 1 – point variants, chromosomal variants and copy number variations.

A point variant is a single base pair alteration that can impact protein structure. Some of these variants can lead to a new codon encoding the same amino acid, meaning that the protein will not undergo any changes [21, 24]. Therefore, these are known as “silent” variants. Other times, the substitution of the base pair results in a new codon that translates into a different amino acid, called “missense” variant if the protein loses its function, or a “nonsense” variant if the new base encodes for a STOP codon, thus creating a truncated protein. The addition/insertion or deletion of one or more base pairs can also occur, leading to drastic loss of function. These are called “frameshift” variants, meaning that it causes a change in the reading frame, introducing or removing unrelated amino acids into the protein [21].

Chromosomal variants, or chromosomal structural rearrangements, can also be responsible for illness. The implication of this rearrangements in gamete production and fertility will be discussed later on.

Copy Number Variations (CNVs) correspond to alterations in the number of copies of specific regions of DNA, either deleted or duplicated. Since CNVs can comprise part or all gene, they are likely to disrupt human normal physiology, and consequently be the cause of a disease [25]. In this class of variants, the expanding trinucleotide repeats should be highlighted. These repeat variants are often unstable (or dynamic) and can differ in size between generations. Moreover, depending on the size of the repeat, different phenotypical traits can emerge [26]. Such diseases frequently present clinical anticipation, which means that the age of onset diminishes throughout generations. There are a number of genetic diseases caused by an expansion of a trinucleotide pair repeat, such as Fragile-X syndrome, HD, Myotonic dystrophy, among others [26].

As referred before, each individual has two alleles for each gene, each one inherited from each parent. These alleles may present variants that may or may not cause illness. These variants are also classified as dominant or recessive. A dominant variant will have a phenotypic impact in the individual, even when only one allele has the pathogenic variant. In contrast, for a recessive disease, a phenotypical expression appears when both alleles present the pathogenic variant. As such, we can distinguish different patterns of inheritance [21]. If the variant affects an autosome, we can distinguish Autosomal Dominant (AD) inheritance from

Autosomal Recessive (AR). If the variant affects a sex-chromosome, then we can discriminate X-linked disorders, that can either be inherited in a dominant (X-linked D) or recessive (X-linked R) pattern, and Y-linked disorders. Mitochondrial DNA is transmitted from the mother to the descendants and can also present variants that will be inherited by the offspring [27]. However, it is important to point out that not all variants are inherited - some occur spontaneously, and not all will have a significant impact on the individuals' health [28].

Table 1. Types of variants and their impact. Some examples of Human Diseases linked to these pathogenic variants.

Class of variants	Type of variant	Description	Example of Human Diseases linked to this variant
Point variant	Substitution	One base is incorrectly replaced during replication	Sickle-cell anemia
	Insertion	One or more nucleotides are inserted during replication, which can lead to frameshift	Beta-thalassemia
	Deletion	One or more nucleotides are "skipped", which can lead to frameshift	Cystic fibrosis
Chromosomal variant	Inversion	One region of a chromosome is cut out, flipped and reinserted	Opitz-Kaveggia syndrome
	Deletion	A region of a chromosome is lost, causing the absence of all genes in that area	Cri du chat syndrome
	Duplication	A region of a chromosome is repeated, resulting in an increase in dosage from the genes in that region	Some types of breast cancer
	Translocation	A region from one chromosome is aberrantly attached to another chromosome	Chronic myelogenous leukemia
Copy Number Variations	Gene amplification	The number of tandem copies of a locus is increased	<i>MECP2</i> duplication syndrome
	Expanding trinucleotide repeat	The normal number of repeated trinucleotide sequences is expanded	Fragile X syndrome, Huntington disease

Adapted from [24].

1.3 Medically Assisted Reproduction

In 1978, Dr. Robert Edwards was responsible for the birth of the first child conceived by *in vitro* fertilization (IVF) [29]. His work led him to win the Nobel Prize in Physiology or Medicine, in 2010, "for the development of the *in vitro* fertilization" technology [30]. Since then, the assisted reproduction technologies (ART) have expanded and impacted the world of reproduction, as it is estimated that 80 million people worldwide are infertile, subfertile or suffer

from viral infections that could spread to the partner while conceiving [31]. In 1992, the first paper concerning Intracytoplasmic Sperm Injection (ICSI) was published, stating that it was possible to achieve a pregnancy with this technology [32].

IVF is based on the incubation of oocytes (retrieved from the woman's ovaries) with the sperm obtained from the partner, in special petri plates [33]. ICSI differs from IVF since it only uses one spermatozoon, that is directly injected into the cytoplasm of the oocyte [32].

ART was at first used in infertile couples, but its use is now open to fertile couples carriers or affected with a genetic disease, with the desire of having a healthy child, by using preimplantation genetic testing (PGT) and embryo selection [34]. These technologies can also allow specific Human Leukocyte Antigen (HLA) matching, in order to conceive a child that could act as a donor for a previous child in need of a stem cell transplant [35]. These indications will be discussed further on.

1.4 Preimplantation Genetic Testing (PGT)

A proof of concept for the detection of genetic disorders in human *in vitro* fertilized embryos, prior to their transfer to the woman's uterus, was published by the group of Alan Handyside in the Hammersmith Hospital in London, in 1989 [34]. This breakthrough was the light of hope for couples with inherited disorders that did not want to see their offspring affected. In this revolutionary paper, five couples with X-linked disorders, such as Lesch-Nyhan Syndrome and Duchenne Muscular Dystrophy (DMD), proceeded with an *in vitro* fertilization, followed by a 2-cell biopsy on day 3 of embryo development, and DNA amplification to perform sex identification, using the at the time newly developed PCR technique. Only female embryos were transferred, and 2 pregnancies were obtained, both with twin pregnancies. After this first success, PGT has progressed enormously.

'PGT' was not the initial term used in this field. Preimplantation genetic diagnosis (PGD) was used when the indication was to look for a hereditary disease that could be transmitted to the descendants - detection of specific variants for disorders such as DMD or Cystic Fibrosis (CF). Preimplantation genetic screening (PGS) does not focus on a specific gene or chromosome, but rather on the screening for chromosomal abnormalities to avoid the present of aneuploidies, particularly in patients with advanced maternal age (AMA) or a history of recurrent miscarriages. Today, PGD has become officially PGT-SR (PGT for chromosomal structural rearrangements) and PGT-M (PGT for monogenic disorders) and PGS refers now to PGT-A (PGT for aneuploidy screening) [36, 37].

PGT involves a multidisciplinary team, comprising physicians, geneticists, technicians, and psychologists. PGT can be performed *in-house*, meaning that the IVF center and the diagnosis center are located in the same institution, or in transport PGT, meaning that fertilization,

embryo culture and embryo biopsy are performed in a satellite IVF center and the biopsied cells samples are then transported to a genetic diagnosis center. After the diagnosis, the transfer is also carried out in the satellite IVF center, as well as the follow-up of the pregnancies and resulting children [38].

1.4.1 Embryo Biopsy

Embryo biopsy is one of the crucial moments in the PGT procedure, and several ways of performing it have appeared throughout the years.

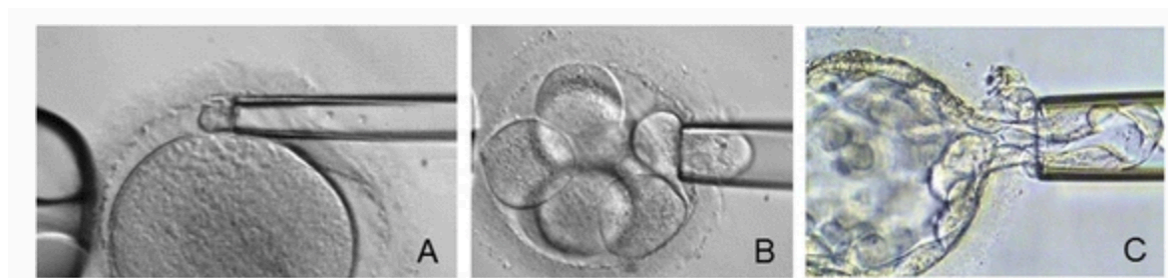


Figure 1 – Types of embryo biopsy. (A) Polar body, (B) blastomere, and (C) trophectoderm biopsy. Adapted from [39].

Polar body biopsy (Fig. 1A) was initially performed in 1990, when Verlinsky and his team suggested that the first polar body might possess crucial information about the maternal contribution to monogenic diseases. This technique allows the transfer of the embryo in the same cycle, eliminating the need for cryopreservation. Removal of polar bodies is expected to have no impact on embryonic development, since the polar body has no function during this process. Also, the technique is minimally invasive, as it only requires a pipette to penetrate the Zona Pellucida (ZP) to retrieve the polar body [40]. Soon enough, Verlinsky team realized that they would need to execute the biopsy on both polar bodies for higher accuracy. A very noticeable disadvantage is that only maternal genetic alterations can be evaluated. Consequently, the diagnosis might be incomplete due to the lack of analysis of the paternal genetic contribution [41].

The most widely used strategy to perform embryo biopsy was the cleavage stage biopsy (Fig. 1B). In this approach, embryos are biopsied in the third day post-insemination, when they reach a 6- to 10-cell stage [42]. The biopsy involves the opening of ZP, so that the blastomeres can be aspirated. ZP opening can be performed using mechanical, enzymatic or laser drilling methods. Initially, embryo biopsy at day 3 was a difficult procedure due to the tendency of blastomeres to adhere to each other. To improve the biopsy procedure, Dumoulin and colleagues developed, in 1998, a Ca^{2+} - Mg^{2+} free medium – the lack of these ions disrupts the tight junctions and cell-to-cell interactions between the blastomeres, facilitating their retrieval [43]. Although one or two blastomeres can be biopsied at this stage, De Vos and coworkers

showed, in 2009, that the two-blastomere biopsy impacted more negatively on further embryonic development than the one-blastomere retrieval [44]. Therefore, it is recommended to only biopsy one blastomere.

The cleavage stage biopsy has been the most common technique for embryo biopsy for more than two decades, although it has some disadvantages. Firstly, the amount of DNA present in a single blastomere is very limited. Furthermore, at this early stage of development, high rates of mosaicism are present, which can lead to false positive or false negative results. However, both maternal and paternal genetic contribution can be analyzed, and the analysis on day 3 also allows a fresh transfer of the embryo around day 5 [38, 45].

Finally, the trophectoderm (TE) biopsy (Fig. 1C) is performed when the embryo achieves its highest stage of development *in vitro*, the blastocyst stage. As embryo culture systems improved over time, PGT centers gradually switched towards TE biopsy, and it is now the most widely used technique [46]. At this point in development, the blastocyst has differentiated parts: the inner cell mass (ICM), the outer TE cell layer and a cavity filled with fluid, the blastocoel (BF) [38]. The TE biopsy protocol may vary but most protocols are derived from the “day 5/6 laser assisted ZP opening and TE biopsy” protocol, that was outlined by Veiga and colleagues, in 1997 [42, 47]. This consists in using laser energy to open the ZP and let the TE cells herniate from the hole, and then excise them with some extra laser shots. The major advantage of this strategy is that the ICM remains intact, thus, this technique is considered safer than the cleavage-stage biopsy. Scott and his team demonstrated that TE biopsy has no measurable impact in embryonic development, while cleavage-stage biopsy severely reduces the embryonic development potential [48]. Another advantage of this approach is, although chromosomal mosaicism is present, the levels appear to be lower in comparison to the levels at cleavage stage. Still, there is a limitation with regard to a fresh embryo transfer, which enforces the cryopreservation of all embryos while waiting for the diagnosis [45].

As cleavage stage and TE biopsy techniques are considered invasive, a lot of research has been done to find a less invasive, but still accurate method. Blastocentesis involves aspirating an aliquot of the BF (Fig. 2). This is based on the current knowledge that the BF contains cell-free DNA. The procedure involves the immobilization of the blastocyst, by a holding pipette, and an oil-filled pipette that penetrates through the TE layer, in the opposite side of the ICM. The fluid is slowly aspirated until the blastocyst has fully collapsed [49]. Clinically, the blastocentesis may represent a less invasive strategy, with some advantages: it avoids the need to remove embryonal cells and the embryo may undergo fewer manipulations since BF aspiration is already applied in routine vitrification (in vitrification, it is necessary to induce a collapse of the embryo in order to avoid ice crystal formation and improve the embryo

survival post-vitrification) [50]. However, this technique has not yet reached the accuracy and reproducibility levels required for routine clinical PGT application.



Figure 2 – Blastocoel fluid aspiration. Adapted from [49].

Another interesting option is the use of cell-free DNA present in the blastocyst culture medium. Capalbo and his team compared blastocentesis samples and spent embryo culture medium samples with TE biopsy samples for PGT-M. Both non-invasive techniques did not provide consistent and fully reliable diagnosis, enhancing the need for protocol optimization [51]. The use of non-invasive technologies would transform the field of PGT, as they are considered much safer for the embryos, but further investigation is required.

1.4.2 Diagnosis technologies

The first PGT cycle was performed in 1989 for five couples in risk of transmitting an X-linked disorder. The procedure involved the amplification of a short Y-specific fragment by PCR to sex the embryos [34]. Although the use of the PCR technique was innovative at that time, it faced several problems, such as failure of amplification and possible contamination. Three years later, nested PCR was used to diagnose CF in embryos, resulting in the birth of a healthy child not carrying the disease. A nested PCR procedure was chosen to increase the level of amplification and concurrently decrease the accumulation of nonspecific DNA products [52]. Nested PCR involves two sequential amplification reactions - the first using a pair of specific primers to amplify the DNA fragment containing the variant, and the second to further amplify the previous fragments with different primers, annealing within the first amplicon [53]. This was an improvement, but still some cells completely failed in amplifying, while in others the amplification of one of the two alleles failed in a random way, a phenomenon known as allele dropout (ADO) (Fig. 3) [54].

Another technique appeared in 1992, to prevent the transmission of X-linked disorders, known as *fluorescent in situ hybridization*, or FISH [55]. With FISH, labeled probes specific for the X and Y chromosomes were used to identify the sex of embryos, allowing sex selection and thus avoiding the transmission of such diseases. FISH was originally proposed as an alternative to PCR methods, and the technique grew to identify CNVs and structural

rearrangements [56]. As FISH was very limited in the number of chromosomes that could be analyzed [57], new genome-wide technologies emerged, such as quantitative PCR (qPCR), microarray-based comparative genomic hybridization (aCGH), and next-generation sequencing (NGS) for analysis of chromosome aberrations (PGT-A and PGT-SR). Within the context of PGT-M, Multiplex-PCR and single nucleotide polymorphism microarrays (SNPa) technologies will be focused.

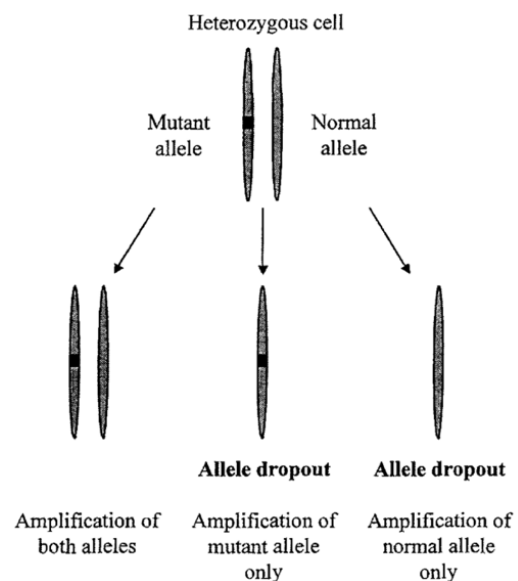


Figure 3 – Representation of ADO phenomenon. Adapted from [53].

○ Multiplex-PCR

In the beginning of the 1990s, due to the great effort to sequence the full human genome, a class of genetic markers was found, today known as Short Tandem Repeats (STRs), spread throughout the entire genome. The STR markers are characterized by a short sequence of two or more base pairs, highly polymorphic, repeated a variable number of times [54].

It is possible to combine several primer pairs in the same PCR reaction mixture, thus creating a Multiplex-PCR protocol. Provided there is no unspecific interaction between the primers chosen, various loci can be amplified at the same time. Therefore, it is possible to optimize primer combinations, and by tagging them with different labels, it is possible to distinguish the different loci amplified [53]. The selection of informative STR markers, located flanking and not too far from the causal genetic variant site is essential. A fully informative STR marker shows different repeat lengths on all four parental alleles following PCR and fragment analysis (informativity testing of genomic DNA samples of the couple). Next, segregation testing is carried out relying on genomic DNA of relatives with known genetic status, to determine which allele co-segregate with the pathogenic variant allele and which allele co-segregates with the normal variant allele. Once segregation or phasing patterns have been established, they can be used to diagnose the embryonic cells. There is still a risk of ADO, but the probability of ADO affecting many or all STR markers is very low, making it a more reliable test than the simplex PCR with genetic variant detection only [54].

Although Multiplex-PCR has a number of advantages, amongst which the low cost of the reagents, it also has disadvantages. The workup, which needs to be done for each family individually, is very time consuming and labor intensive, since it is needed to identify informative markers for the condition and establish segregation in each particular family [54].

If the high-risk haplotype is determined during the workup, for embryo diagnosis it is applied an indirect testing method, that only comprises the selected STR markers. Alternatively, the detection of the variant by minisequencing methods can be added to the clinical protocol for haplotype confirmation in case it was not possible to determine the high-risk haplotype, which is a common situation in families carrying a *de novo* variant, or in case no family relatives are available to establish segregation [58].

○ **SNPa**

The implementation of whole genome amplification (WGA) methods was a technical breakthrough, that unlocked several options for new approaches in embryo diagnosis. This strategy aims to increase the amount of DNA present in samples with low quantity of genetic material, such as blastomeres [59]. Several methods were implemented throughout times. The development of multiple displacement amplification (MDA) method was truly revolutionary. It relies on an isothermal reaction (at 30 °C) performed by a DNA polymerase with strand-displacement activity, such as *Phi29* ($\Phi29$). Random exonuclease-resistant primers will anneal to the denatured DNA and, after elongation and displacement of the new double-stranded DNA, additional priming events will keep occurring, resulting in a network of branched DNA strands over 10 kb (Fig. 4) [60].

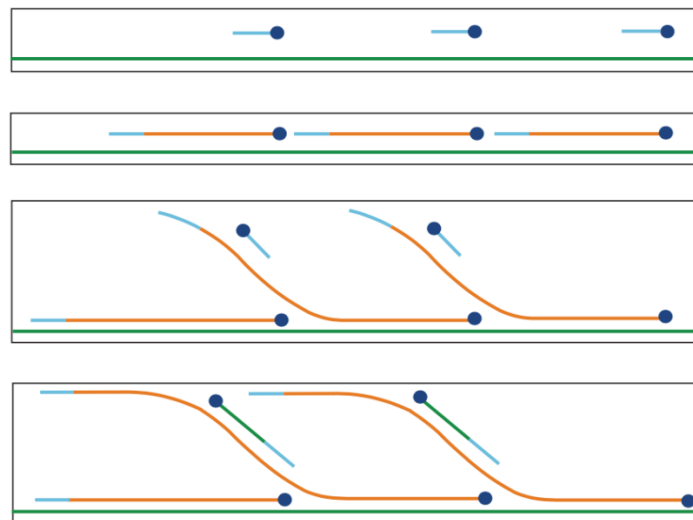


Figure 4 – Overview of the process of MDA. Random hexamers (blue line) bind to the denatured DNA (green line). The $\Phi29$ polymerase (blue circle) extends the primer until reaching newly synthesized double-stranded DNA (orange line). The enzyme displaces the strand and continues the polymerization reaction, while other primers bind to the new DNA. Polymerization in the new strand begins, forming a branched structure. Adapted from [60].

The improvement of WGA methods enabled the introduction of more comprehensive approaches, such as SNPa. A SNP array consists in a DNA microarray with a high density of oligonucleotide probes. These probes allow the genotyping of hundreds of thousands of selected single nucleotide polymorphisms (SNPs) across all chromosomes, in a unique

reaction. A SNP is a genome position where it is possible to find two distinct nucleotide residues (or alleles), that appears in a significant portion of the human population. As SNPs are mostly biallelic, alleles are named A and B, creating the possible genotypes homozygous AA or homozygous BB, and heterozygous AB [45, 61]. The procedure and analysis of a SNP array will be discussed in the methods section. The major advantage of this technique is the reduction of waiting time for the couple and the laboratory workload, since there is no need to search for STR markers near the gene, design primers, test and select informative genetic markers. All SNPs are available on one array, ready to use [45]. Other advantage is its capability to detect a wider range of chromosomal abnormalities, allowing the combination of PGT-M and PGT-A. However, it is quite expensive per sample [56].

1.4.3 Embryo transfer and Cryopreservation

As the golden standard methodology for embryo biopsy was the day 3 biopsy, there was enough time to test the embryos and perform a “fresh embryo transfer” on day 5 or 6; the surplus genetically transferable embryos could be cryopreserved. However, a fresh embryo transfer was not recommended for certain patients, such as patients in risk of ovarian hyperstimulation syndrome (OHSS). In these cases, it is possible to apply a “freeze-all” strategy, that consists in vitrifying all suitable embryos and the transfer is done in a deferred cycle. In this case, embryos are biopsied and tested, and only the genetically transferable embryos are cryopreserved. It has been shown that the clinical outcome of a frozen embryo transfer is superior to a fresh embryo transfer in patients with OHSS [45, 62].

On the other hand, a TE biopsy also requires a freeze-all strategy, due to the time necessary to perform the diagnosis [45]. In this case, the embryos biopsied at day 5 or day 6 are immediately cryopreserved. Genetic testing is performed and, once the results are ready, the genetically suitable embryos remain cryopreserved until the transfer, while the non-suitable embryos are destroyed or saved in another cryopreservation tank for research purposes, depending on what was previously agreed with the couple.

The first systems used to cryopreserve embryos were based on a slow-freeze approach, followed by a rapid-thaw. This freezing technique presented several problems: as it was based on a slow change from a liquid to a solid state, the common occurrence of ice crystals could disrupt the intracellular morphology, which led to low rates of success [62]. Vitrification overcame the majority of these problems by using a high concentration of cryoprotective additives and ultra-rapid cooling, which lowers the risk of crystallization. Therefore, vitrification is considered superior to slow-freezing methods in terms of survival rate, both in cleavage-state embryos and blastocysts [63].

1.5 Legal Framework

The scientific advances in the field of reproduction and embryo selection exposed the necessity for legal boundaries. In Portugal, up to 2006, there was no legal regulation concerning ART or PGT. In 2006, a National Council for Medical Assisted Reproduction (CNPMA) was created. The mission of this Council is to advise about all ethical, social and legal issues of ART [64]. The Portuguese legal framework is updated regularly to fit the new advances in science, with the version from July 2019 being the latest update of this legislation.

This law states that ART are a subsidiary method and not an alternative method of procreation, which can only be used in diagnosed cases of infertility, for treatment of a severe disease, or to avoid transmission of a genetic disease to the unborn baby (article 4. °). Any married or similar status heterosexual couple or two-woman couple or even any women (independently of their sexual orientation or civil status) are allowed to resort to ART (article 6. °). Comparing to the Europe scenario, 11 countries restrict PGT to heterosexual couples (Albania, Bosnia and Herzegovina, Czech Republic, France, Italy, Lithuania, Poland, Slovakia, Switzerland and Turkey), and only 5 allow these techniques for single woman and homosexual couples (Belgium, Malta, Romania, The Netherlands and the UK) [65].

It is well stated that these techniques cannot be used to enhance certain characteristics of the unborn baby, although two exceptions are allowed - PGT with HLA matching and sex selection, if there is a high chance of transmitting a sex-linked genetic disease for which a targeted genetic test is not available (article 7. °).

It is forbidden to create embryos for research purposes through ART. However, scientific embryo investigation is legal with the purpose of embryo diagnosis or therapy, of improving ART, of building a stem cell bank for transplantation programs or for any other therapeutic reasons. Therefore, it is legally allowed to carry out scientific research on embryos that cannot be transferred or cryopreserved: cryopreserved, surplus embryos for which there are no parental projects ahead and embryos with severe genetic anomalies following PGT diagnosis. This only applies after the parents have signed an informed consent, in which they clarify these embryos' destiny (article 9. °).

Chapter 5 of this law focusses on PGT; article 28 clearly states that PGT has the purpose to identify embryos that are not affected with a severe genetic abnormality before being transferred to the women' uterus. It is not allowed to report the carrier status of the embryos for AR disorders. In the absence of a legal definition for "severe genetic abnormality", the CNPMA applies that terminology for diseases that can cause significant suffering and/or premature death [66]. It is possible to do aneuploidy screening on embryos that are going to be transferred, to lower the risk of chromosomal aneuploidies. In the European context, this type of screening is not acceptable in 11 countries (Bosnia and Herzegovina, Malta, Denmark,

France, Germany, Hungary, Lithuania, Norway, Slovenia, Sweden and The Netherlands) but it is acceptable in Portugal, when fulfilling several criteria (defined by CNPMA) [66].

Finally, article 29 states that PGT is available for families with genetic abnormalities that can cause death or severe disease, when there is a high chance of transmitting it to the descendants. All the specific medical indications are determined by good clinical practice and appear in national and international recommendation documents.

1.6 Ethical concerns

Most of the ethical issues surrounding PGT are centered on the rights of the embryo, the future fetus and the soon-to-be-person entity. In today's society, there is a large protection not for life itself, but for quality of life. However, it is not easy to determine if the suffering of a disabled life justifies the rejection of life itself [67]. The moral status of the embryo is, for that reason, one of the most debatable issues.

Throughout time, several concerns to PGT arose, since this technique is centered in creating and selecting embryos, while discarding others. For instance, people in favor of the genetic humanity theory will oppose to the idea of creating and destroying embryos, due to the belief that any living structure that possesses human DNA has a full moral status [68, 69]. Other theories include the implantation theory, that states that the moral status of the embryo is achieved during implantation, the embryogenesis theory, that links the moral status of the embryo to the moment when the embryo is morphologically different from any other animal embryos, and the Singer's theory, that support that the moral status will be achieved during organogenesis, as this is the moment when the embryo becomes sentient [68]. All these theories have arguments pro and con, which are still in debate, making "the moral status of the embryo" a constant evolving concept.

In a different point of view, the use of PGT for embryo selection offers the couple the option of avoiding termination of pregnancy, after pre-natal diagnosis techniques (PND), such as amniocentesis or chorion villus sampling (CVS). By using IVF and PGT, these couples could increase their chances of having an unaffected offspring comparing to the changes of having it through natural conceiving methods [70]. It is necessary to note that PND techniques are still advisable to confirm the diagnosis obtained in the embryos.

Another discussible use of PGT is for screening of susceptibility diseases. The ethical issue is to whether it is justified for a couple to go through IVF and PGT to screen embryos for a disease that could happen later in life, but it is not 100% penetrant. The burden of having a child that has a high chance of developing a severe disease (as cancer) in the future, and for which the offspring will be monitored during its life, and may undergo preventing therapies, such as prophylactic surgery, can cause a lot of pain and suffering, both in the couple and in

the child [70]. Therefore, the use of PGT in these cases may be justified by the risk of developing the disease in the future.

A similar ethical issue occurs in PGT for late-onset disorders, such as Alzheimer's Disease or HD, but with a twist. In this case, there is an additional concern about the ability of the affected parent to raise the child. These late-onset disorders are inherited as AD with 100% penetrance, which means that the parents know they carry the affected gene or are at risk of carrying it. If their wish is to have an unaffected child, they could undergo PGT for embryo selection, reducing the risks of transmitting the disease, but creating a child that might lose one of the parents while still being dependent on them [70].

In a scenario that there is an affected grandparent with a late-onset disease, such as HD, and if the parent doesn't want to know if he/she inherited the pathogenic variant, two options can be offered: non-disclosure testing and exclusion testing [71]. For the non-disclosure strategy, embryos are tested and only those not affected by the disease are transferred, while ensuring that the parent does not know his/her genetic status. However, this testing method is not recommended since it requires extreme confidentiality and, if no unaffected embryos are available, doctors may feel coerced into conducting unethical behavior (for instance, fake embryo transfer) [45, 71, 72]. The exclusion test also acknowledges the right of the parent not to know whether he/she is affected by the disease [58], but in this case, embryo diagnosis is performed selecting for the haplotype of the unaffected grandparent and against the haplotype inherited from the affected grandparent (Fig. 5). Therefore, there is a 50% chance that the parent has inherited the affected haplotype, but there is no possibility of knowing what haplotype was inherited. There is also an ethical issue in the method because there is a chance perfectly healthy embryos will be discarded if the parent did not inherit the mutant haplotype.

PGT for HLA matching is another debatable use of this technique since it is used to give birth to another child for stem cell transplantation (from cord blood or from bone marrow), to cure an affected sibling. This can be offered in two different situations – firstly, in cases where the existing child has an acquired disorder, such as leukemia, and secondly, in cases where the existing child has a monogenic disorder and HLA typing is combined with PGT-M to avoid another affected child and simultaneously create a child HLA compatible with the diseased one. Due to ethical concerns, some countries only allow HLA matching when combining with PGT-M. Other issues arise in this discussion, for instance, the reasons why the parents are bringing a new child to the world, the risk for the female partner (because she would probably need to undergo several ART cycles) and other risks for the offspring. It is important to note that the whole process has risks, starting in the embryo biopsy [73].

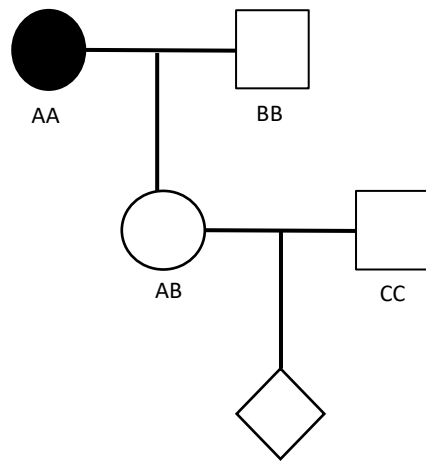


Figure 5 – Schematic scenario of an exclusion test request. Embryos will be tested for the B haplotype, and against the A haplotype. Embryos that inherited the A haplotype will not be transferred, even though there is a 50 % chance that the embryo will be unaffected.

It is not uncommon to hear that, with the technological advances in the field of reproduction and embryo selection, there is space for eugenic manipulation [74]. Another controversial use of PGT would be the screening of embryos for nonmedical traits, such as sexual orientation, height, intelligence, among other characteristics. This scenario is not completely implausible although not yet possible. Such traits are very complex, and their genetic basis is still unknown – it may involve a large number of genes, with many loci, so selecting for these traits is not feasible with the current technology [75].

1.7 Indications for PGT

Today, PGT is used for testing for monogenic disorders (PGT-M), for chromosomal structural rearrangements (PGT-SR) and for aneuploidies (PGT-A). Besides all criteria specific for each indication group, it is recommended by the European Society of Human Reproduction and Embryology (ESHRE) PGT Consortium that PGT should only be offered and applied when it is possible to perform it, from a technical point of view, and that the test applied is reliable [76]. Also, there are several factors, such as safety issues, female age, impossibility to retrieve male or female gametes, body mass index (BMI) and other contraindications for IVF, that should be considered as exclusion criteria. Furthermore, PGT should be carefully evaluated if one of the soon-to-be parents suffers from a severe physical or psychological problem, either linked to the disease that will be tested or due to other conditions [76].

PGT-M is a term currently used to refer to the testing of DNA pathogenic variant(s) causing monogenic disorders, for which the disease-causing loci has been unequivocally identified [76]. The variants are classified as: (1) benign; (2) likely benign; (3) uncertain significance; (4) likely pathogenic and (5) pathogenic [23]. When weighing in if the couple should receive PGT treatment, only couples with variants in class 4 or 5 should be included [76].

PGT-M combined with HLA matching is mostly requested by couples that have a child affected by a monogenic disease and wish to have another child to act as a donor for stem cell transplantation, but also not affected with the same disorder. The first case reported for PGT-M combined with HLA matching was for Fanconi Anemia, a congenital disorder that often requires a stem cell transplant [77]. When all other clinical options have already been studied and exhausted, PGT for HLA matching is also a morally acceptable option for couples who have an affected child with an acquired malignant haematological syndrome [76]. Normally, these children will only recover with a stem cell transplant. Obviously, this solution is only acceptable when the parents intend to love and care the newborn in the same way as the affected child [71].

PGT-SR was developed for couples that are not able to conceive naturally or have a high risk of miscarriage due to inherited unbalanced products of chromosomal rearrangement [76]. In this category, we can distinguish different kinds of chromosomal structural rearrangements, such as reciprocal and Robertsonian translocations, insertions, deletions, duplications and inversions, that can be inherited or occur as *de novo* variants [78]. When the rearrangement is familial, PGT-SR enables the identification of the chromosomally unbalanced progeny at an early stage of development.

PGT-A is, among the three types of PGT, the most debatable one. PGT-A has several aims, such as (1) increase the pregnancy rate, (2) decrease miscarriage rate, (3) decrease the malformation rate, (4) decrease the multiple pregnancy rate, and (5) decrease the number of pointless ART treatments [36]. There are already some inclusion indications listed, such as AMA, recurrent implantation failure (RIF), recurrent miscarriages (RM) and severe male factor (SMF). The definition and criteria for each factor is established by each PGT center [76].

1.8 Trajectory of PGT

The procedure for PGT is normally long (Fig. 6). It often starts with a request from the couple that is evaluated by a multidisciplinary team. For this, it is necessary to collect personal information of the couple, such as names, date of birth, pedigree of the family, among others. It is also important to understand if any members of the family are available to provide blood samples [79]. Reports on male and female reproductive history and fertility status are required before starting PGT, as well as the results of the genetic testing and karyotypes [76].

The type of request (PGT-M, PGT-SR or PGT-A) is another item to be evaluated. For PGT-M, it is imperative to know the disease, its pattern of inheritance and if the request includes HLA matching. Then, it is essential to know the variant and how is it classified (for PGT-M, only class 4 or 5 variants are considered). For an AD disease, the future of the parent affected with the condition should be assessed. For PGT-SR, it is needed to check what kind of structural

abnormality is present in the soon-to-be parents and if the breakpoints of that abnormality are well defined or if there is a need to define them. Lastly, for a PGT-A procedure, the couple must fulfill certain criteria, as stated above [79].

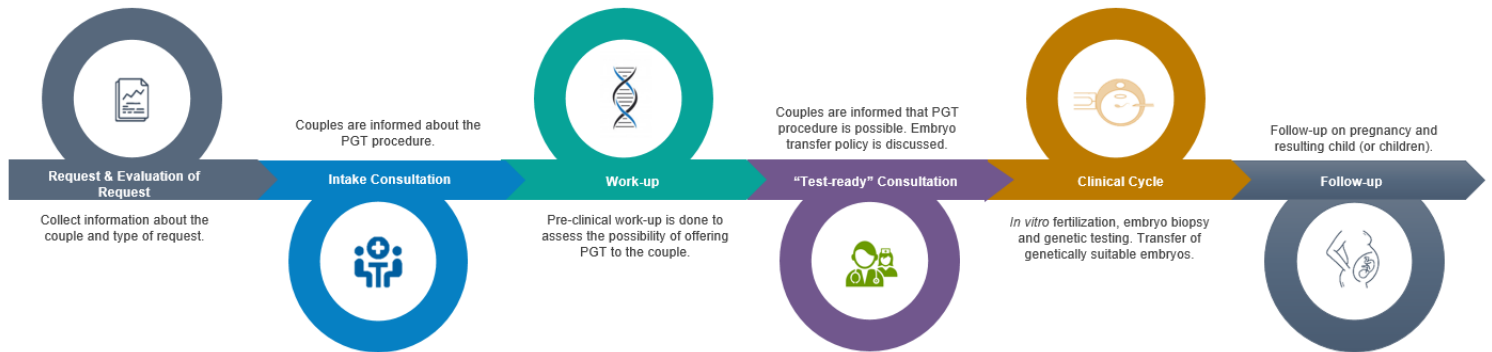


Figure 6 – The trajectory of PGT. Couples fill a request for PGT, that is followed by an intake consultation. A pre-clinical workup is done to assess the feasibility of PGT. Couples are called in to a “test-ready” consultation and proceed to the clinical cycle. After transfer of genetically suitable embryos, pregnancy is monitored, as well as the children that were born.

The intake consultation is a combined appointment with a clinical geneticist, a PGT counselor and a reproductive medicine specialist. A psychologist might also be present under certain circumstances. During this session, couples are informed about the different aspects of PGT treatment, including average success rates and misdiagnosis rates. Couples are also invited to sign an informed consent document which details information about the procedure, the risk of false-negative diagnosis, possible risks regarding the transfer of mosaic embryos, advice on prenatal testing as a follow-up method, and issues about the destiny of embryos that are not considered genetically suitable for transfer [79].

Genetic counseling starts at this point, but it should be available throughout the procedure. The genetic counselor is going to verify all molecular reports of the couple and the family members that were available (also identified as index patients) to check if it possible to offer PGT to the couple. Then, blood samples are collected for the pre-clinical workup. In case the mother-to-be is affected with the disease, it may be necessary to obtain a medical report stating that there is no contra-indication for a pregnancy [79].

After the workup is done, the couple comes back for a “test-ready consultation”, with a clinical geneticist and a specialist in reproductive medicine. In this session, embryo transfer policy will be discussed again - during embryo testing no changes are allowed in what was agreed. In PGT-M combined with HLA matching cases, it is important to discuss what is the destiny of non-compatible embryos that are not affected with the disease. In the case of X-linked recessive disorders, it is required a decision on the destiny of the female carrier

embryos. After this consultation, it is possible to proceed to the clinical cycle. In this next section, we are going to focus on the following stages: workup, clinical cycle, and follow-up.

a. Workup

The first step in the workup is to choose the technology that is going to be used for the diagnosis of embryos. In this project, we are only going to analyze how to perform the diagnosis for PGT-M requests (with or without HLA matching).

The Multiplex-PCR technique was the most widely used strategy for many years and it is usually offered for couples that have a common variant for which an optimized single cell Multiplex-PCR protocol is available - for instance, the common variant c.1521_1523delCTT (also known as p.Phe508del), that causes CF. For Multiplex-PCR, a set of STR markers are chosen from public databases and combined to co-amplify different amplicons in all four parental alleles, allowing allele discrimination and making it possible to identify the potential genotypes for the embryos [58]. Using SNPs it is also possible to determine the segregation using informative SNPs. As explained before, SNPs are mostly biallelic but are less powerful markers, since three informative SNPs are equivalent to one informative STR marker. The most powerful informative SNP is the one that is unique in the WT allele, enabling the distinction of unaffected embryos by using heterozygous SNPs [58].

There are several methods to perform allele discrimination, such as fragment length analysis, restriction enzyme digestion, minisequencing, among others. The fragment length analysis is carried out via capillary electrophoresis (CE) and it is based on the different migration patterns of different amplicons, according to their molecular weight or size. Fragment length is used for allele discrimination in case of STRs and for (likely) pathogenic variants involving one or few base pairs deletions or insertions. Fragment length differences cannot be applied to detect single nucleotide substitutions, so other methods are used. The restriction enzyme digestion is performed with an enzyme capable of recognizing certain DNA sequences near the variant site which will cleave either the normal or the pathogenic allele. The PCR amplification is followed by a purification step and restriction enzyme digestion, and restriction fragments are visualized after fragment length analysis [58]. Alternatively, minisequencing which is based on Sanger sequencing methods can be used. This reaction also requires a purification step after the first PCR reaction. After purification, a new PCR procedure is performed, using a specific unlabelled minisequencing primer (either a forward or a reverse primer), designed to anneal directly adjacent to the mutation site. This minisequencing primer will be extended with a single fluorescent dideoxy nucleotide (ddNTP), that is complementary to the target site. Each ddNTP is labelled with a different fluorochrome, which allow allele distinction without the need of sequencing the entire amplicon (Fig. 7) [58, 80].

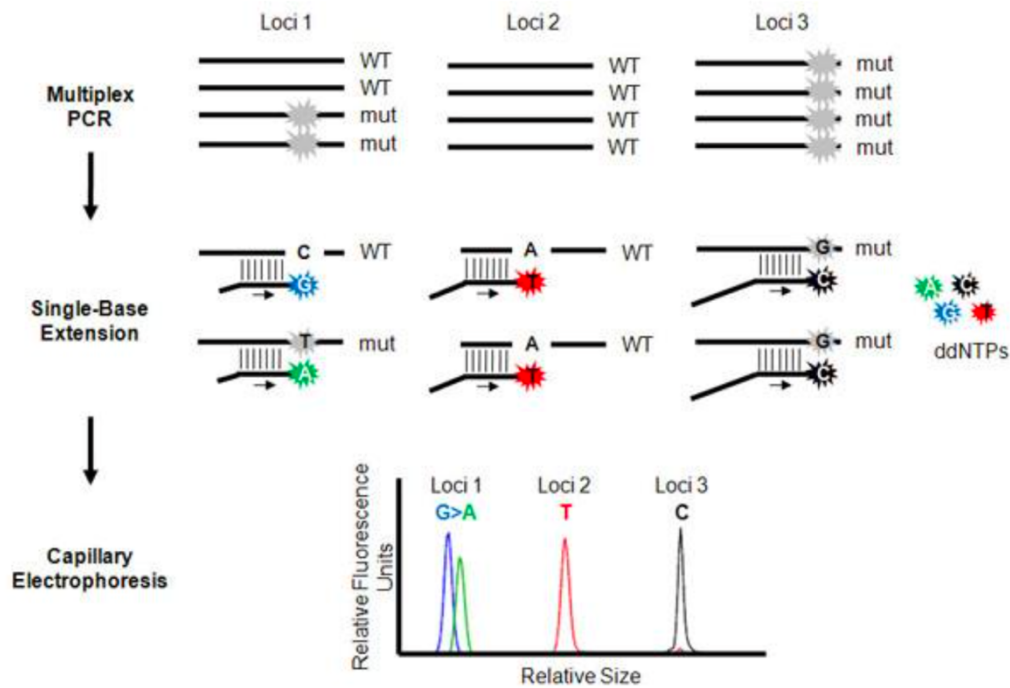


Figure 7 – Overview of minisequencing technique. After a Multiplex-PCR and a purification step (not shown), a single-base extension reaction allows the introduction of one ddNTP directly adjacent to the mutation, allowing the distinction of alleles by capillary electrophoresis.

The test strategy for the couple is then defined based on the results of the informativity/segregation analysis. When referring to PGT-M, we can consider three main strategies - (1) amplification of STR markers, with or without the variant, in a Multiplex-PCR, (2) WGA followed by the amplification of STR markers, with or without the variant detection and (3) WGA followed by SNP_a. The first strategy is obviously more time consuming since it is necessary to develop a test and validate it on single cells. The second approach is more generic since it is possible to skip validation of PCR reactions on a single cell level. However, both strategies cannot provide a comprehensive view of the genome. WGA followed by SNP_a allows genome-wide haplotyping and chromosome copy number typing, being a more comprehensive methodology. It is important to emphasize that this strategy does not leave enough time to perform a fresh embryo transfer, a problem that is overcome by embryo cryopreservation [58].

As referred before, the detection of the variant is not always included in the test strategy. In the first and second approaches, the detection of the variant is included in case of a *de novo* variant, in case no family members are available (segregation will only be determined on the embryos during the first PGT cycle in both cases) or in case the selected clinical protocol (KLI) contains its detection (for instance, most of the KLI for CF contains the common mutation c.1521_1523delCTT). For the SNP_a approach, the variant detection is included in case of a *de novo* mutation, in case no family members are available (segregation has to be determined on the embryos during the first PGT cycle in both scenarios) and in case

there are not enough SNPs for diagnosis accuracy. In these cases, additional STR markers and/or the variant can be tested.

b. Clinical cycle

After the work up is finished, the couple can initiate a clinical cycle. For that, the female needs to undergo an ovarian reserve test to determine the optimum stimulation protocol, and a serological test to check for infection diseases. The sperm of the male also needs to be analyzed, especially when the male carries a hereditary condition that might reduce spermatogenesis (as CF, polycystic kidney disease, myotonic dystrophy, among others) [79]. After oocyte retrieval, ICSI is performed to avoid paternal contamination from remaining sperm attached to ZP. The denudation procedure (careful removal of cumulus cells) minimizes the risk of maternal contamination from residual cumulus cells [81].

The embryos are cultured and grown until biopsy time. Traditionally, human embryos have been cultured in simple salt solutions, balanced with added carbohydrates, such as glucose, pyruvate and lactate [82]. Today, it is known that these mediums lacked many important components to conserve embryo's physiology and health, such as aminoacids (AA) [83]. EDTA (ethylenediaminetetraacetic acid) also has an important role in the culture medium due to its chelating activity – capability of “sequestering” metal ions, weakening their reactivity. It is essential to maintain a constant volume to avoid osmotic perturbations, that might result in swelling or shrinking of the embryonic cells, as well as a pH between 7.2 and 7.4 [84]. Other factors also need to be present in the medium, such as macromolecules (like proteins as serum albumin) [84], vitamins (vitamins C and E have been shown to reduce oxidative damage [85]), growth factors [86] and antibiotics (preventing bacterial contamination [87]).

The biopsy should be performed in the minimum time possible, on an inverted microscope with heated stage and three-dimensional micromanipulators and microinjectors, positioned on antivibration pads. As discussed earlier, the main approaches for embryo biopsy are PB biopsy, cleavage-stage/blastomere biopsy or blastocyst/TE biopsy. After biopsy, cells are collected into small reaction tubes for testing, a procedure known as tubing. Before transfer to the reaction tubes, cells should be washed at least twice with the help of a sterile transfer pipette. Tubing can be performed in PBS or in lysis buffer, depending on the protocol of the PGT center [81]. After biopsy, cells undergo genetic testing to discern the genetically suitable from the non-suitable embryos for transfer.

c. Follow-up

Pregnancy of these women should be carefully observed since there is an increased risk for placental disorders, such as placenta previa (the placenta covers completely or partially the

female's cervix) and cesarean section, comparing to women that conceived naturally [88]. PGT children, when comparing to IVF conceived children, do not seem to present a higher risk of preterm birth, low birth weight and longer neonatal admission time [88].

Generally, couples undergoing PGT are advised about the risk of misdiagnosis and the necessity of a prenatal test to confirm diagnosis accuracy. All options should be presented to the couples, including invasive prenatal tests, such as amniocentesis or CVS, and non-invasive techniques, as cell-free fetal DNA. If the couple refuses prenatal diagnosis, another option could be discussed – cord blood sampling, which would be done at birth to confirm the genotype or karyotype [76].

The follow-up of the children after PGT is very important since it is an invasive technique. As mentioned before, couples undergoing PGT need to go through an IVF treatment with ICSI, which may increase the risk of birth defects. PGT has extra risks due to embryo biopsy, another invasive procedure. However, the risk of malformation in PGT children (2.5 %) seems comparable to the risk in ICSI children (1.67 %) and naturally conceived children (absolute increased risk of 2 %) [89, 90]. Concerning the birth weight, ICSI new-born children present a higher incidence of low birth weight (< 2500 g) than PGT babies, but the rates of very low birth weight (< 1500 g) are comparable between these two groups [91].

Follow-up in childhood and adolescence has been done in small cohorts. A study published in 2016 showed that children that reached 6 years old, after PGT-M and PGT-SR, do not present any significant differences on body composition measurements or blood pressure, comparing to naturally conceived children [92]. A more recent study revealed that five-year-old children that were born after PGT also had a normal growth, health, and motor development, when compared with naturally conceived children [93]. These studies, although encouraging, highlight the importance of follow-up of children to monitor PGT safety.

1.9 Accreditation and Quality Management

“Accreditation is a formal recognition that an authoritative body gives to a laboratory/department/center when it demonstrates competence to carry out defined tasks and involves all aspects of management, along with technical requirements” [94]. IVF and PGT centers should be accredited, even if it is not legally binding, which allows these centers to establish, accomplish and maintain the highest quality standards.

It is also recommended that IVF and PGT centers have a quality management system in place, to provide consistent quality. This system should contain 4 main tasks: quality planning, quality assurance (QA), quality control (QC) and quality improvement. Moreover, QA and QC are prerequisites to most accreditation protocols [94]. An important element for PGT centers is the participation in external quality assessment schemes.

2. Aim

This project focusses on cases of transport PGT-M between the Genetics Department of the Faculty of Medicine of the University of Porto and the Center of Medical Genetics of the UZ Brussel, a collaboration in place since 2006. The Genetic Unit in Porto is responsible for the PGT requests and for collection of genetic reports and blood samples (both from the couples and family members). The IVF satellite center, also in Porto, carries out embryo fertilization, culture, biopsies, and sample collection, which are then sent to the PGT center in Brussels, where the workup and embryo diagnosis takes place. For many years, a day 3 biopsy followed by a single-cell Multiplex-PCR was carried out. Only recently day 5/6 biopsy followed by WGA and SNP_a was implemented.

The main aim of this project is to compare day 3 biopsy/targeted testing with day 5/6 biopsy/genome-wide testing method for specific parameters (such as number of embryos tested, number of embryos genetically suitable, efficiency of the genetic test, contamination and ADO rates, clinical outcome, among others). Special cases, such as double indications or *de novo* variants will also be considered for comparison. A secondary aim is to compare this cohort of transport cycles with other datasets (*in-house* and ESHRE PGT consortium).

3. Methods & Materials

3.1 Patient population & study design

A collaboration between the Faculty of Medicine of Porto's University and the Center of Medical Genetics of the UZ Brussel center is in place since 2006. During this time, 284 couples have requested PGT treatment. Information for each couple was collected retrospectively, from 2006 to 2021, to construct a database. This database is divided into seven sections: couple information, disease description, family member(s) information, pre-clinical workup, clinical cycle(s), pregnancy and birth. All items included in the database are listed on Table 2.

The disease description section contains information about the OMIM (Online Mendelian Inheritance in Man) number of the disease and the gene. OMIM is a very extensive online database on all known Mendelian disorders and over 15,000 genes [95]. The inheritance of the disease – AD, AR, X-linked D or X-linked R – and the disease status, both of the female and male – affected, carrier or unaffected – are also stated. The variant and its classification based on public databases, such as ClinVar, are included, with the proviso that, since 2015, only variants from class 4 (likely pathogenic) and 5 (pathogenic) are acceptable for PGT [23]. Variant type – single nucleotide polymorphism, insertion, deletion, inversion, insertion and deletion (indel), duplication, contractions - was similarly included.

The family member section includes information about the family member(s) availability, its degree of kinship – son/daughter, father or mother of the female/male, aunt of female/male, cousin of female/male, brother/sister, nephew/niece – the variant and their status, analogously to the previous section.

Table 2. List of parameters collected retrospectively and included in the database.

Couple information	Clinical cycle	Number of fetuses heartbeats
Name of female	Number of cycle	Number of fetus sacs
Female's date of birth	Date of oocyte retrieval	Pregnant
Name of male	Age of female	Number of babies
Consanguinity	Purpose (PGT/accumulation of embryos)	Pre-natal test
	Number of oocytes	Expected deliver date
Disease Description	Number of inseminated oocytes (ICSI)	Delivery date
Name of disease	Number of 2PN	Term (weeks)
OMIM number of disease	Number of embryos biopsied	
Inheritance pattern	Number of cells biopsied	Birth
Disease status of female	Type of cells biopsied	Birth Weight (g)
Disease status of male	Number of embryos tested	Postanalysis (yes/no)
Gene	Day of biopsy	
OMIM number of gene	Number of genetically untransferable embryos	
Variant	o Affected/HLA not compatible	
Variant Classification	o Abnormal	
Variant type	o Number of embryos without diagnosis due to failure of amplification	
HLA matching request (yes/no)	o Number of embryos without diagnosis due to failure of contamination	
De novo variant (yes/no)	o Number of embryos without diagnosis due to failure of recombination	
	o Number of embryos without diagnosis due to failure of ADO	
Family member	o ADO rate	
Family member availability (yes/no)	o Contamination rate	
Name	Number of genetically transferable embryos/HLA compatible	
Degree of kinship	Number of embryos cryopreserved	
Variant	PGT outcome	
Disease status		
Work-up	Pregnancy	
Date of intake	Date of transfer	
Approach	Number of embryos transferred fresh	
Inclusion of variant (yes/no)	Number of embryos transferred frozen	
Date of report	bHCG (positive/negative)	
Result (green/red light)	Number of fetuses	

The workup segment includes the date of intake, date of the report and the result – green or red light. The possible approaches are ‘short-PCR’, meaning that there were already protocols with enough STR markers ready *in-house*, ‘long-PCR’, meaning that a new protocol had to be developed, which includes the selection of suitable STR markers and/or minisequencing for variant detection, or ‘SNPa’. The variant may be detected by minisequencing in any of these approaches depending on several factors – its availability *in-house*, if it is a *de novo* variant and/or if no DNA samples from family members are available. A ‘retesting’ option is also possible – usually, after 2 years, PCR protocols need to be retested to assess if the reaction conditions are still valid.

The clinical cycle - either for accumulation of embryos or PGT - is initiated with an ovarian stimulation and the first parameter collected is the date of oocyte retrieval. The age of the female is calculated at that time. The number of oocytes that undergo insemination (ICSI) and the number of oocytes that are normally fertilized are counted. At this point, embryos can

be cryopreserved at day 2 (or rarely on day 3), or continue to develop and be biopsied, either on day 3 or day 5/6. The type of cells biopsied – blastomeres or TE cells – depends on the approach chosen previously. The biopsied cells are transported to the genetic diagnosis center, undergo diagnosis and each embryo is classified in genetically non-transferable – affected, abnormal or without diagnosis due to failure of amplification, ADO, recombination or contamination – or genetically transferable. Empirically, embryos from transport PGT present higher levels of ADO and failure of amplification, so calculation for ADO and no amplification rates is done by dividing the number of embryos with no diagnosis due to ADO or failure of amplification by the total of embryos tested. The number of embryos cryopreserved also depends on the approach chosen – day 3 biopsy allows enough time to complete the diagnosis and cryopreserve only genetically transferable embryos; following day 5/6 biopsy, all embryos are cryopreserved and, after diagnosis, the embryos suitable for transfer are kept cryopreserved until being transferred. Several options for PGT outcome are taken into consideration:

- No OPU – no oocytes are available, so PGT cycle is canceled.
- No biopsy – embryos do not present enough quality to be biopsied, so PGT cycle is canceled.
- Fresh ET – fresh embryo transfer after PGT.
- No ET – no embryo transfer after PGT due to lack of genetically suitable embryos for transfer or lack of enough embryo quality.
- Freeze all – PGT is not followed by a fresh transfer; however, all genetically transferable embryos, after a day 3 biopsy, are cryopreserved and/or all embryos, after a day 5/6 biopsy, are cryopreserved.
- Freeze all without cryo – PGT is not followed by a fresh transfer and there is no genetically transferable embryos or not enough quality embryos to be cryopreserved.
- Embryo freeze D2/3 – embryos are frozen on day 2 or 3 of development, prior to the biopsy procedure.
- FRET – frozen embryo transfer.

In the pregnancy section, date of transfer and number of embryos transferred (fresh or frozen) is collected. The levels of bHCG (beta Human Chorionic Gonadotrophin) hormone are measured and classified as positive or negative. Pregnancy can occur or not, but other scenarios might arise (Table 3) [96]. The expected delivery date is calculated by adding 38 weeks to the date of transfer, and the term of the baby is calculated in weeks by the difference of the date of birth and the date of transfer, plus two weeks. If the couple decided to have pre-natal or post-natal tests was also taken into consideration, as well as the birth weight, in grams.

Table 3. Possible scenarios for transfer outcome, other than pregnancy itself.

Biochemical Pregnancy	A pregnancy diagnosed only by the detection of bHCG in serum or urine.
Clinical Pregnancy	A pregnancy diagnosed by ultra-sonographic visualization of one or more gestational sacs or definite signs of pregnancy; in addition to intra-uterine pregnancy, it includes a clinically documented ectopic pregnancy.
Ectopic Pregnancy	A pregnancy outside the uterine cavity, diagnosed by ultrasound, surgical visualization, or histopathology.
Elective Termination	Interruption of a pregnancy before the 20 th week of gestation at the woman's request, for reasons other than maternal health or fetal disease.
Ongoing	The pregnancy had completed over 20 weeks of gestation.
Stillbirth	The death of a fetus prior to the complete expulsion or extraction from the mother after 28 completed weeks of gestational age.

Adapted from [96].

3.2 Workflow

Patients file the request for PGT and have the intake consultation in Porto. Blood samples are collected from the couple and, if available, from relevant family member(s). DNA is extracted from white blood cells by an Automatic Extractor Prepito, from Perkin-Elmer, and sent to the PGT center in Brussels. Pre-clinical workup is performed by using either direct or indirect Multiplex-PCR or SNP_a, and a report with workup result is given to the couple.

The method chosen for the pre-clinical workup was not always standardized and has evolved over time (Fig. 8). It depends both on the test methodology (targeted *versus* genome-wide testing) and on the strategy (indirect testing based solely on genetic markers *versus* a direct testing which includes detection of the (likely) pathogenic variant) [45]. In our center, transport PGT between 2006 and 2016 was only performed by Multiplex-PCR, with a 2-cell biopsy at day 3 of embryo development. Since 2016, SNP_a was introduced for couples with a *de novo* variant or for couples that had no family members available but was gradually extended to all other couples. Since 2019, Multiplex-PCR development is almost null.

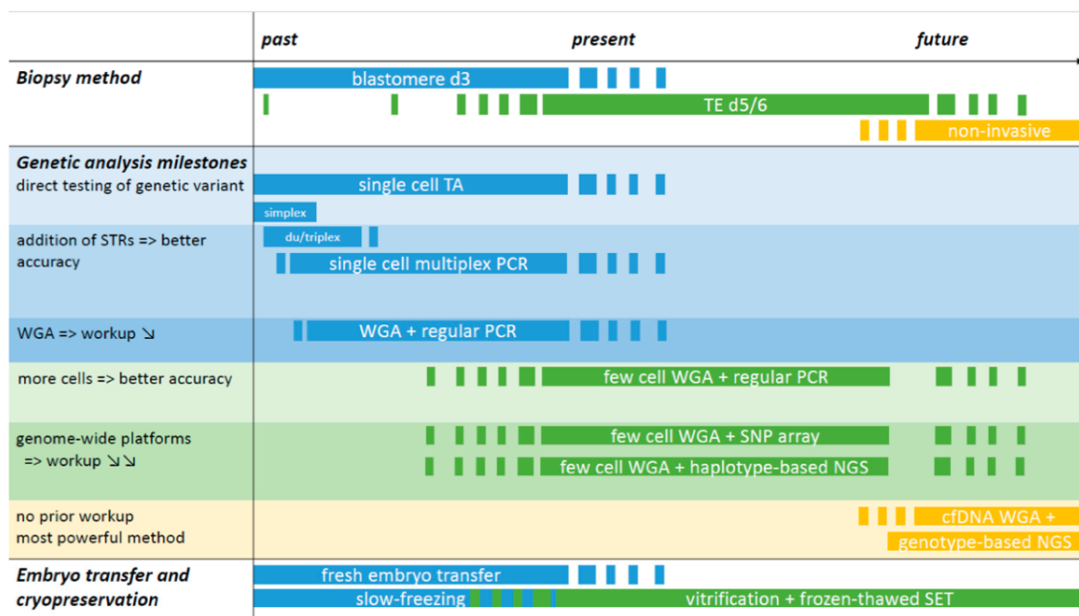


Figure 8 – Overview of PGT-M timeline. In the past, Multiplex-PCR was the golden standard for PGT and it is still performed today, although in fewer families. WGA was a technical milestone, allowing the increase of DNA amount. WGA followed by SNP array is now the most widely used technique in PGT-M. Adapted from [45].

After a green report stating that it is possible to perform embryo diagnosis, the couple can proceed to a clinical cycle. Ovarian stimulation, oocyte retrieval, artificial insemination by ICSI, embryo culture and embryo biopsy are performed in the IVF satellite center in Porto. The biopsied cells are then sent to Brussels for analysis. When the diagnosis is ready, the results are sent back and the genetically suitable embryos can be either transferred or cryopreserved. The pregnancy and resulting child (children) are followed in Porto.

Biopsied cells must be tubed and store on dry ice for transportation over long distances. Alkaline lysis buffer (ALB), composed of 200 mM NaOH and 50 mM 1,4-dithiothreitol (DTT), dissolved in water, is used for blastomere tubing for single cell PCR tests, whereas PBS (1x) (phosphate buffered saline) is used for tubing TE cells for SNP array analysis. ALB and PBS (1x) aliquots are provided to external labs (in this case, to the IVF center in Porto) - for their transport, aliquots are stored in a transportation box. Detailed information about the stability during transport and storage in the laboratory is provided in Table 4.

Blastomere tubing for single cell PCR test is done in 0.2 mL PCR tubes, which are kept on ice during the whole procedure. 2.5 µL of ALB is distributed to each PCR tube, which are labeled unambiguously. Tubing is carried out in a horizontal flow with a mouth controlled pipetting system in the following way: rinse the stripper tip 3 times with a PBS + BSA (bovine serum albumin) solution and aspirate a small amount of the liquid. Visualize the blastomere in the biopsy dish under the microscope and aspirate the cell. Transfer the cell into the first washing droplet of the corresponding labeled row and continue with two more washing steps.

Aspirate the cell from the third washing droplet and transfer it to the corresponding PCR tube, while visualizing this under the microscope. It is important to minimize the amount of PBS + BSA that is transferred to the PCR tube, since BSA can act as an inhibitor of the amplification reaction. Lastly, transfer $\pm 0.5 \mu\text{L}$ of the remaining PBS + BSA from the stripper tip into the PCR tube that serves as blank and visualize this transfer under the microscope. These blanks are collected together with each tubed blastomere and are processed together with the cells during the amplification reaction. Note the status of the blastomere before (blastomere was lysed or not) and after transfer (blastomere was visualized or not).

Table 4. Conditions of stability during transport and storage in the lab for ALB and PBS aliquots.

Reagent	Stability during transport	Storage in the lab
ALB	○ ALB aliquots are stable while frozen. ○ Shipped on dry ice	Temperature: -20°C Time: up to 6 months
PBS	○ PBS is stable for 48h in temperatures between 4 and 15°C. ○ Shipped between cooling elements	Temperature: 4°C Time: up to 1 year

TE cells tubing for SNP α is also done in 0.2 mL PCR tubes, which are kept on ice through the whole process. In these, distribute $4.0 \mu\text{L}$ of PBS (1 x). The procedure differs from the blastomere tubing only after the transfer of the TE cells to the third washing droplet, that only contains PBS, whereas the two firsts washing droplets contained PBS + BSA. After transferring the TE cells to the third washing droplet, rinse the stripper tip 3 times with only PBS (1 x) and aspirate a small amount of PBS. Visualize the TE sample in the third washing droplet and aspirate the TE sample, transferring it into the corresponding PCR tube while visualizing this under the microscope. Note the transfer (TE sample was visualized after transfer or not) and how the TE sample looked like (many/few cells, shape, color, or other specific characteristics of the biopsied sample). A negative control (NC) is taken in 1 per 4 embryos, and no blanks are collected.

PCR tubes with single cells or TE samples, blanks and NCs are stored at -20°C or -80°C ; minimum storage time at -20°C is 30 minutes before preparation of the transportation box. This minimum storage time is crucial: the small volume should be frozen (and must remain frozen during transportation) in order to prevent the volume to disperse all over the tube. Place the tubes in a rack, wrap up with parafilm to ensure that the tubes stay in the rack and put a

cover on the rack; write the name of the patient(s) on the cover and wrap up the rack plus the cover again with parafilm. Put this on dry ice in the transportation box.

3.3 Multiplex-PCR

STRs, also known as microsatellite regions, are non-coding repetitive DNA sequences of 1 to 6 bases, with a high degree of polymorphism and widely distributed all over the human genome. Multiplex-PCR is based on the co-amplification of several STR markers within the same reaction, an adaptation of the conventional PCR reaction. Given the nature of this reaction, it is crucial to properly select the primers used, so that all are evenly amplified, but also to obtain amplicons of different lengths to enable their distinction after CE. For many years, it was necessary to develop and validate Multiplex-PCR protocols for each family, which includes the design of STR primers and/or minisequencing primers.

3.3.1 Development and Validation

As mentioned before, all Multiplex-PCR protocols were designed based on a specific family (Fig. 9). Normally, 4 to 5 STR markers in each flanking region of the gene of interest (both 3' and 5') are chosen from published papers or by an *in-silico* selection from public databases [58]. Primers are designed, ordered and, when ready, are tested individually, on a simplex PCR done in genomic DNA (gDNA concentration should be 10 ng / μ L). This is important to observe if all STR markers amplify well, at the same annealing temperature, assessing if they can be combined in a Multiplex-PCR reaction.

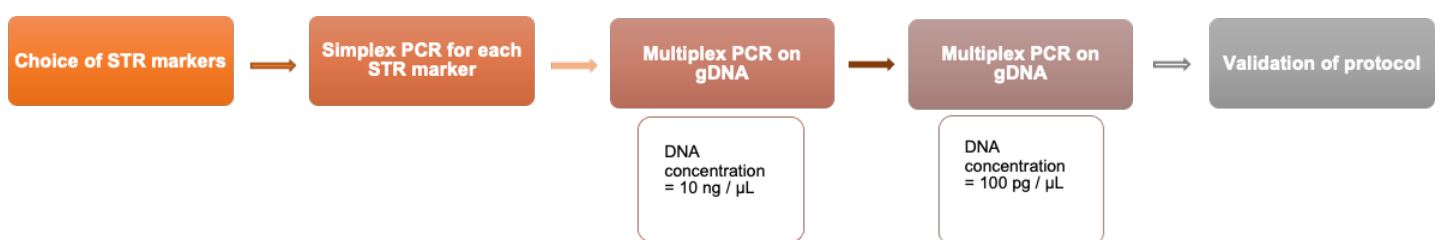


Figure 9 – Overview of the process of development and validation of a Multiplex-PCR protocol.

If the results of the simplex PCRs are considered good, the STR markers are tested on the gDNA samples of the family, so that is possible to access how polymorphic they are for the couple of interest. Not all STR markers may be selected for the next phase due to lack of high polymorphism. Also, it is important to observe balanced peaks, otherwise it might be necessary to adjust the reaction conditions and/or the concentration of the primers.

All selected STR markers are again tested, at a DNA concentration of 100 pg / μ L. At this stage, due to the low DNA amount, the number of cycles of the amplification reaction are raised. The protocol may still need some adjustments - sometimes it is important to add other reagents, like the Q reagent, that helps amplifying CG-rich regions. If the results are considered good, then it is possible to proceed to the final step - the validation.

Validation of a Multiplex-PCR protocol is done in four sets of 12 single cell and 12 blanks each set. For each amplicon, amplification, ADO, and contamination rates are calculated, and when they achieve the pre-defined threshold (over 95 % of amplification and less than 5 % of ADO and contamination) the Multiplex-PCR is considered ready to be applied in the clinic.

3.3.2 STR primer design

STRs are selected both upstream and downstream of the gene, 5' and 3', in a maximum distance of 1Mb from the gene of interest. Primer sets (both forward, Fw, and reverse, Rv, primers) are designed largely with the use of software tools. Some primer characteristics are stated on Table 5.

The target site for primer annealing must be properly selected. It is vital to check for the presence of SNPs among the primer annealing sites, because SNPs can negatively influence PCR amplification and lead to no amplification. On a suitable reference sequence database, it is also checked if the primer set is unique, meaning that it would bind exclusively to the target sequence. After these steps, one of the primers is labeled fluorescently and ordered to a company [97]. It is important to note that only about 85 % of the genome can be specifically amplified due to the presence of repetitive sequences, gene families (set of similar genes, resulting from the duplication of an original gene and normally with similar biochemical functions) and pseudogenes (nonfunctional segments of DNA that resemble functional genes).

3.3.3 Minisequencing primer design

The minisequencing reaction is based on Sanger sequencing methods. It uses a specific unlabelled minisequencing primer (either Fw or Rv primer), designed to anneal directly adjacent to the mutation site. Primers can be developed according to the sense or antisense strand, to meet the design requirements. Also, SNP check is a requirement.

This minisequencing primer will be extended with a single fluorescent dideoxy nucleotide (ddNTP), that is complementary to the target site. Each ddNTP is labelled with a different fluorochrome, which allow allele distinction without the need of sequencing the entire amplicon.

Table 5. Primer characteristics (primer length, amplicon length, melting temperature, GC content, 3' terminus, label and repeats), recommended optimal scenario and justification.

Characteristic	Optimal scenario	Reason
Primer length	Between 18 and 22 bp	Long enough for high specificity and short enough to easily match the DNA template; difference of length within a primer set should not be higher than 3 bp.
Amplicon length	Between 80 and 320 bp	---
Melting Temperature (T_m)	Between 52 and 65 °C	T_m increases the chance of nonspecific annealing; differences of T_m within a set of primers should be as small as possible (< 3 °C).
GC content	Between 40 and 60 %	---
3' terminus	Last 5 bases should contain at least one G or C	Should not have a T or an A as the last base given its least specific binding; it is also not recommended to use 3 consecutive C's and/or G's as last bases, that could result in hairpin loops and/or primer dimers.
Label	5' label primer	Optimal resolution and fragment detection – typically Fw primer is labelled, but if it starts with a G, the Rv primer is labeled
Repeats	Maximum number of di-nucleotide repeats is 4	A repeat (di-nucleotide occurring many times consecutively) should be avoided because they can misprime - anneal to locations in DNA which might be present other than the intended location.

Adapted from [97].

3.3.4 Multiplex-PCR, analysis, segregation and interpretation

During workup, gDNA samples from the couple and the available family member(s) are diluted to a DNA concentration of 10 ng / μ L. In a horizontal flow, prepare all primer mixes (dilute, in water, each primer set to a previously determined concentration). Prepare the reaction mix with all primer mixes, water and master mix. A PCR master mix normally contains a DNA polymerase, dNTPS, magnesium chloride ($MgCl_2$) and buffer. As mentioned before, some protocols might include other reagents, as Q reagent. Distribute the reaction mix in small PCR tubes and, in another pre-PCR room, add the gDNA dilutions. Immediately place the samples in the PCR machine to start the reaction.

For a clinical cycle, the protocol has some differences – the transported blastomeres must stay for 30 min at - 24 °C; when defrosted, blastomeres are lysed for 10 min at 65 °C in

a thermocycler, and the gDNA of (only) the couple is diluted to 100 pg / μL (the reference is not included at this step). All the procedure is done on ice. Also, tricine is added to the reaction mix, due to its power to neutralize ALB.

PCR takes place in 3 steps, all sensitive to temperature: denaturation, annealing and extension. Denaturation takes place at 94 °C, which converts the double-stranded DNA molecules to single stranded. The primers anneal to the complementary regions of single stranded DNA and are then extended by a DNA polymerase [97]. Annealing and extension temperatures, as well as the number of cycles of the PCR reaction, were optimized during the development and validation of the protocol.

The resulting fragments are analyzed using an automated capillary electrophoresis device (Applied Biosystems 3730xI DNA Analyzer). The PCR products are loaded on a plate with a matrix standard (either GeneScan ROX 500 or GeneScan LIZ 1200). During CE, the PCR products enter the capillary by an electrokinetic injection and are separated by size. Analysis is performed in a GeneMapper 5 software (Applies Biosystems by Thermo Fisher Scientific) and STR markers are classified as fully informative, partially informative, and non-informative. An example for an AD disorder is provided in Table 6. Only fully informative markers provide a clear distinction of all possible embryo genotypes, as well as enabling the detection of ADO, contamination, and recombination issues. Partially informative markers do not distinguish all genotypes for an embryo and are less powerful for ADO, contamination, and recombination detection. A non-informative marker will not be useful for embryo diagnosis, because it is not possible to clearly distinguish an affected from an unaffected embryo [58]. For a more accurate diagnosis, the presence of unique alleles, meaning alleles present only in one of the parents, is very important.

Table 6. Example of STR markers informativity classification for an AD disorder, in which the male is affected, and the female is unaffected. The pathogenic allele is indicated with the symbol (*).

Affected male partner	Unaffected female partner	Informativity
124-126*	120-122	Fully informative
124-126*	120-126	Partially informative
124-126*	126-126	Partially informative
124-126*	120-124	Partially informative
124-126*	124-124	Partially informative
126-126*	Any genotype	Non-informative

Adapted from [58].

If the protocol requires the mutation detection by minisequencing, a purification step is needed between the two reactions (Fig. 10). When the PCR products are purified, the minisequencing reaction is performed using the SNaPshot™ Kit from Applied Biosystems. It is necessary to prepare a primer mix, by diluting the minisequencing primer in water to the desired concentration, and a reaction mix, with the primer mix and the SNAPmix (which contains the enzyme and the fluorescently labeled ddNTPs). All the steps should be performed on ice. Usually, 6 µL of reaction mix are distributed to each PCR tube and 2 µL of DNA is added. After the PCR reaction, the fragments are analyzed by CE.

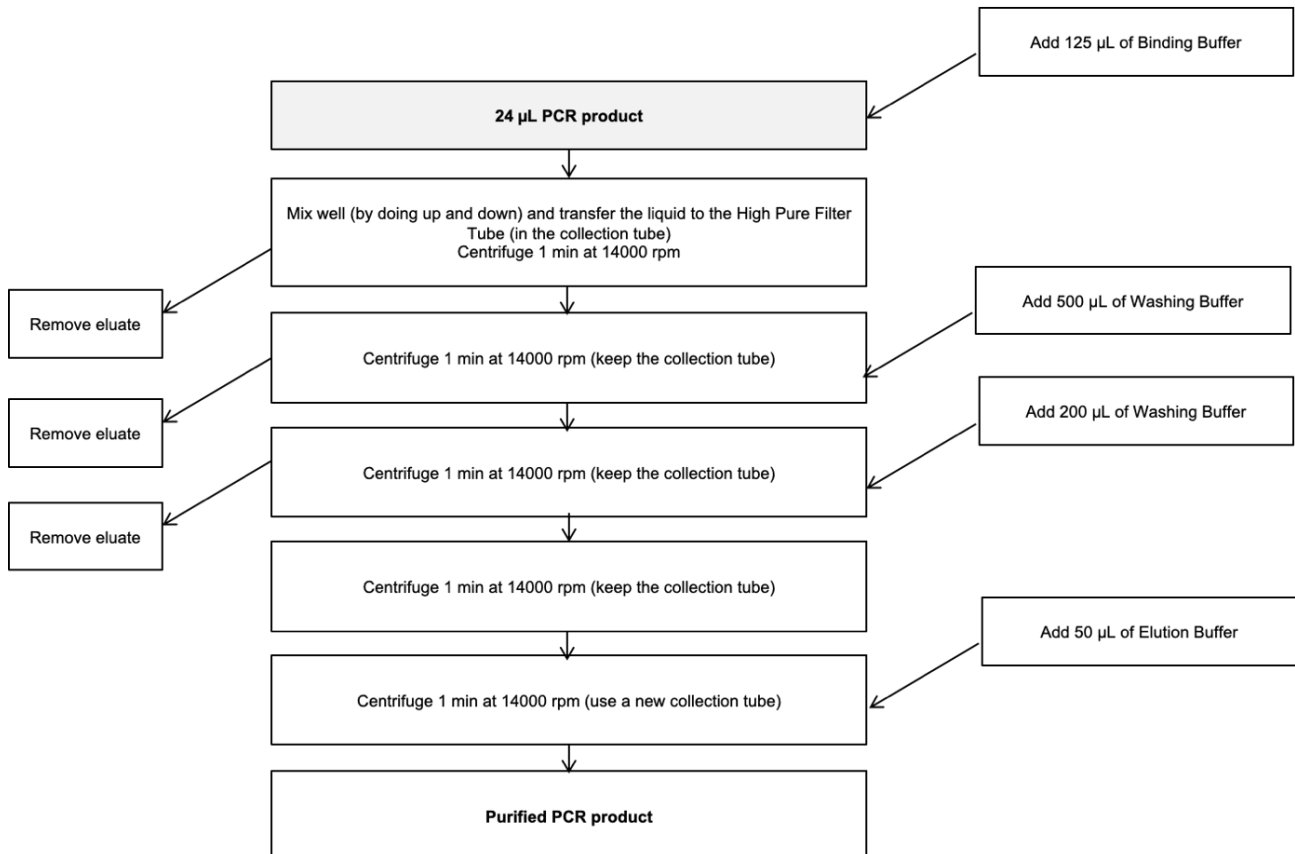


Figure 10 – Overview of the purification process for a minisequencing reaction.

3.4 WGA by MDA

WGA is performed on TE biopsied cells using the MDA technique (Repli-G Cell WGA & WTA Kit, by Qiagen). Frozen samples are transported from Porto to Brussels in dry ice. After thawing, 3 µL of D2 buffer (lysis buffer) is added to each PCR tube and cells are incubated in a thermocycler (Gene Amp PCR Systems 9700, Applied Biosystems by Thermo Fisher Scientific) for 10 min, at 65 °C (lid temperature: 105 °C). D2 buffer is prepared by adding DTT and DNA Lysis Buffer (DLB). After incubation, immediately add 3 µL of STOP solution. After

that, add 40 μL of Amplification Master Mix (containing 9 μL H_2O , 29 μL of buffer and 2 μL of the enzyme) to each sample and proceed to a 2-hour incubation at 30 $^{\circ}\text{C}$.

For visualization of DNA, a 0.8 % agarose gel is prepared by pouring 50 mL GelRed 1x TBE (Tris/Borate/EDTA) DNA gel stain. After adding 0.4 g of agarose, heat it on the microwave for 1 minute, stirring every 30 seconds. Pour it into the container and let it settle. When ready, load 2 μL of sample and 8 μL of loading buffer in each well, and 3 μL of ladder on the side well. The run takes 40 min at 125 V and 180 mA, in an Electrophoresis power supply – EPS 200. The gel is then photographed at a UV-light camera, by Vilber Lourmat to observe the amplification, and printed with a Video Graphic Printer Up-897 MD, by Sony.

3.5 SNPa

SNPa is a long procedure, subdivided into eight steps, normally done throughout 2 days. Although long and laborious, it can be done for four BeadChips at one time, which makes 48 samples (12 per BeadChip). An overview of the procedure is detailed on Figure 11. The BeadChips are read in an Illumina iScan.

The results are analyzed on Bluefuse Multi Software, by Illumina. When the array is scanned, SNP genotypes are called based on the total fluorescence and the ratio of hybridization intensities of A and B (allele frequencies). Similar to STR marker analysis, SNPs are classified as informative or non-informative. An informative SNP occurs when one of the members of the couple is homozygous (either AA or BB) and the other one is heterozygous (AB). The use of a reference (a child, a grandparent, ...) allows the phasing of the parental haplotypes. By default, the mother haplotype is named M1M2, the father haplotype is P1P2, and the reference haplotype is M1P1. Haplotypes produced by karyomapping are relative to the reference. Blue and orange represent the paternal and maternal haplotypes, respectively, inherited by the reference. Red and green are the paternal and maternal haplotypes, respectively, that were not inherited by the reference (Fig. 12) [98]. With genomic DNA samples, call rates of 98 % or more are generally achieved. However, for MDA products such as TE samples, call rates are normally lower, in the 75 – 95 % range. For embryo analysis, only call rates above 85 % are considered, with a miss-call value inferior to 5 %.

For embryo analysis it is important to consider “key” and “non-key” SNPs. Key SNPs are those for which the phasing cannot be altered by ADO, whereas non-key SNPs are those that are most likely to be accurate but may also include any erroneous phasing results caused by ADO (Table 7) [98]. During the workup it is necessary to find 6 key SNPs both 5' and 3' of the gene of interest, in the WT haplotype. If this condition is not met, it is compulsory to add to the diagnosis strategy STR marker analysis.

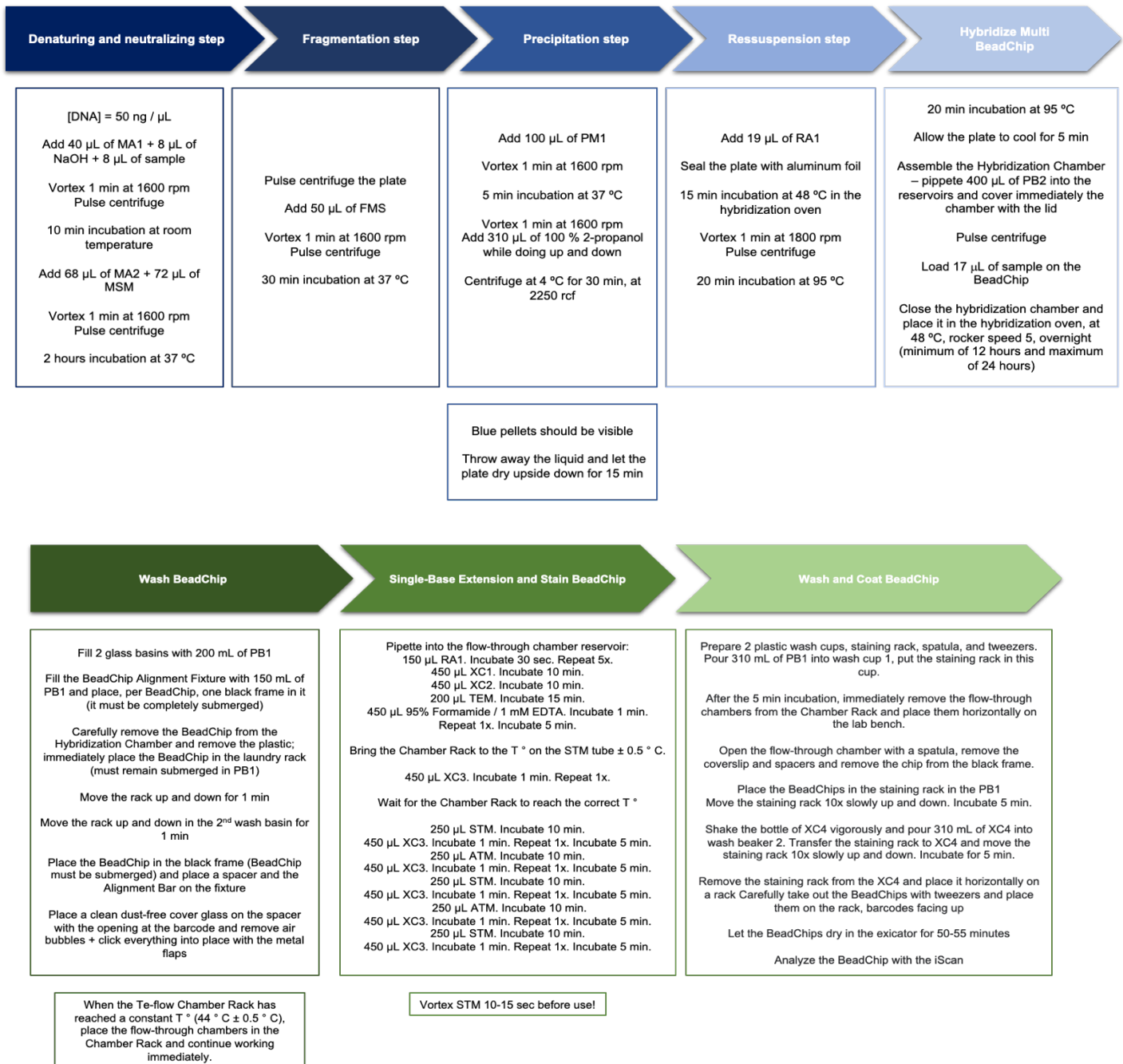


Figure 11 – Overview of the SNP protocol. Steps performed at day 1 are detailed in blue boxes, while in green boxes are the details for steps performed at day 2.

The raw SNP data yields genotype data for PGT-M as well as chromosome copy number data, allowing for concurrent analysis of PGT-A. As such, SNP can reveal the presence of aneuploidies, polyploidies and uniparental disomy [99]. For copy number analysis, two measures are important: the log R ratio and the B allele frequency (BAF). The log R is the $\log_2$ transformed value of the normalized intensity of the SNP. If the log R is zero, it means that there are two copies of each chromosome, thus the embryo is euploid. However, if any deviations are observed, aneuploidies, such as monosomies or trisomies, might be present. The BAF represents the total of SNPs that contains the B allele. BAF values of 0, 0.5 and 1

represent a normal copy number but aberrations will cause a decrease or increase of the total intensity and allele frequencies.

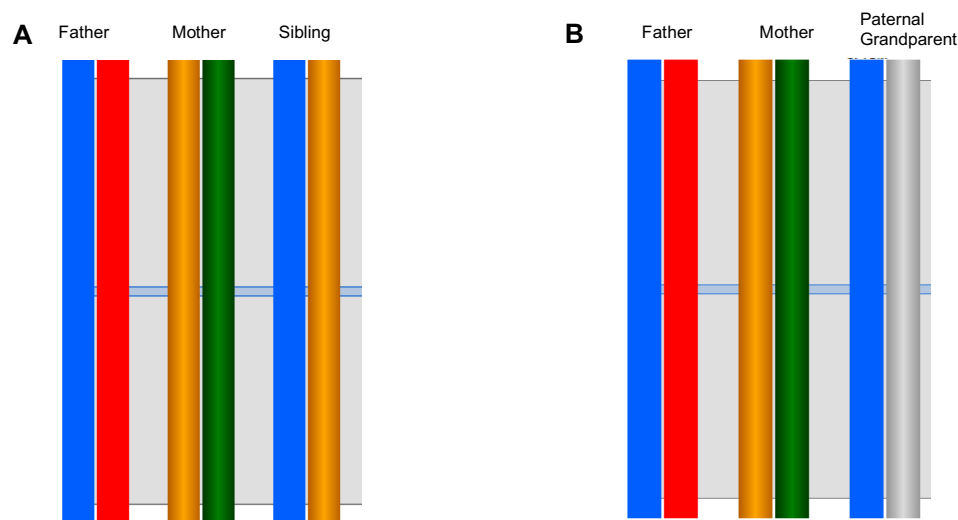


Figure 12 – Representation of the mother, father and reference haplotypes done by Bluefuse Multi Software. **A** – The reference (M1P1) is a sibling and inherited the blue (P1) and orange (M1) haplotypes from the parents. **B** – The reference is a paternal grandparent – the father inherited the P1 haplotype, in blue; the grey haplotype is the one that was not inherited.

Table 7. Example of SNP informativity classification and “key” and “non-key” classification. The informative SNP is indicated in bold blue.

Informative parent	Father	Mother	Embryo
Informative SNPs for father	AB	AA	AB = key AA = non-key
	AB	BB	AB = key BB = non-key
Informative SNPs for mother	AA	AB	AB = key AA = non-key
	BB	AB	AB = key BB = non-key

Adapted from [98].

3.6 Ranking of embryos and transfer policy

Embryo ranking is done differently depending on the test strategy and the type of inheritance of the disorder. For PGT-M diagnosis, embryos are ranked as genetically transferable or not transferable (Table 8). Besides being affected, embryos with no diagnosis due to ADO, contamination, no amplification and/or recombination, also justifies not transferring these embryos. Abnormal/aneuploid embryos are neither considered for transfer.

For transport PGT with Multiplex-PCR, two blastomeres are biopsied for a more accurate diagnosis. In case one of the cells does not show amplification, the diagnosis is given with only one cell. If the diagnosis of both cells shows discordant results, the embryo will not be considered for transfer.

After genome-wide methods of diagnosis, the final ranking is determined by the results of karyomapping obtained in Bluefuse software, supplemented with an *in-house* developed algorithm for whole chromosome copy number detection. SNP_a at a single or few cells level results have a lot of background noise due to WGA pitfalls and, consequently, demand particularly well-developed algorithms for data interpretation. A multidisciplinary team has agreed on this transfer policy, based on current available literature data.

Some studies have shown that embryos with whole chromosome mosaic aneuploidy may result in healthy babies [100]. However, the implantation chances of mosaic aneuploid embryos are lower and the risk for miscarriage is higher, when comparing to euploid embryos. Therefore, embryos are categorized in 3 ranks – transferable with priority rank 1, transferable with priority rank 2 and not genetically transferable (Table 9).

The associated misdiagnosis rate for PGT-M is estimated to be lower than 1%. The misdiagnosis rate for whole chromosome copy number detection is estimated to be lower than 5%. Findings of unknown technical or clinical significance (including mosaicism) are not reported. Reporting on chromosomal copy number is possible in case of good quality results, but there will be no result for aneuploidy in case of lower quality results.

Table 8. Embryo ranking for PGT-M diagnosis, either after a targeted testing (Multiplex-PCR) or wide-genome testing (SNP_a).

Disorder	Affected embryo	Unaffected embryo	Carrier embryo
AD	Not transferable	Transferable	---
AR	Not transferable	Transferable	Transferable
X-linked D	Affected male embryos are not transferable	Transferable	Carrier female embryos are not transferable
X-linked R	Affected male embryos are not transferable	Transferable	Carrier female embryos are transferable

Table 9. Embryo ranking for PGT-A after a wide-genome testing (SNPa).

Embryos genetically transferable - priority rank 1	Embryos genetically transferable - priority rank 2	Embryos genetically not transferable
Euploid embryos. Embryos with mosaic (< 25 % of cells) aberrations (whole chromosome or segmental).	Embryos with mosaic (> 25% and < 75% of cells) aberrations (whole chromosome or segmental) with a low risk to yield liveborn affected children. Embryos with no result for PGT-A.	Embryos showing aneuploidy (whole chromosome or segmental) in all cells (> 75 % of cells). Embryos with mosaic (> 25 % and < 75 % of cells) aberrations with a high risk to yield liveborn affected children (whole chromosome or segmental) - disomy/trisomy for chr 8, 9, 13, 16, 18, 21 and X or mosaic monosomy/disomy X.

3.7 Statistical analysis

For statistical analysis, we relied on IBM SPSS Statistics 27 for MacOS (SPSS Inc.). An exploratory analysis of the data was performed to assess if the parameters in the study followed a Gaussian distribution by employing the Kolmogorov-Smirnov and the Shapiro-Wilk Normality tests. The results of the normally distributed variables are presented as mean $\pm$ standard deviation, while the results of the non-normally distributed variables are presented as median (interquartile range).

To evaluate the association between different variables, the Spearman's rank correlation test was used. An r value between 0.60 and 0.79 was considered a strong correlation, and an r value over 0.80 was considered a very strong correlation. Comparisons between two groups were performed with the Mann-Whitney U test. Comparisons between more than two groups were performed with the Kruskal-Wallis test. A value of $p < 0.050$ was considered as statistically significant. Clinical outcomes were calculated based on what was previously described by Zegers-Hochschild *et al.* [96].

PGT transport data were compared with *in-house* PGT data, from the Center of Medical Genetics of the UZ Brussel. A similar database was already available. Only cycles with a 2-blastomere biopsy and TE biopsy between 2015 to 2019 were included for comparisons.

4. Results

From January 2006 until February 2021, 284 Portuguese couples requested PGT treatment in transport service. An overview of the results is presented in Figure 13.

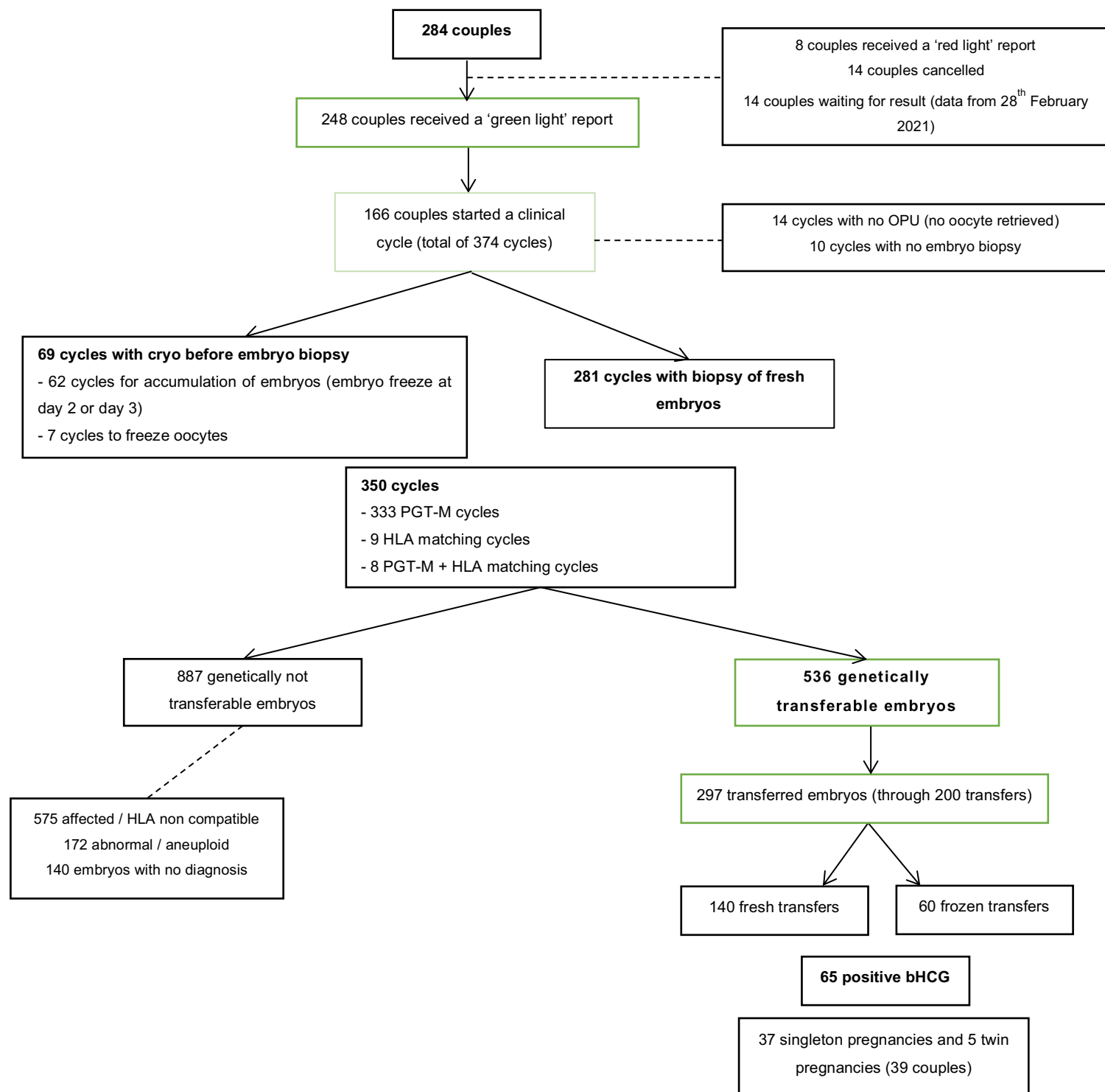


Figure 13 – Flowchart showing the details from PGT requests to clinical cycles. Retrospective data from January 2006 until February 2021.

4.1 Example of a couple workflow

The procedure of PGT is normally long and extensive. To demonstrate the normal procedure of PGT, this section focusses on an example of the PGT workflow. In 2016, a couple requested PGT-M treatment in transport service for Familial Adenomatous Polyposis (FAP; OMIM number of disease: 175100), due to a *de novo* variant in the *APC* gene, on chromosome 5 (OMIM gene number: 611731). FAP is an AD disorder, and the male partner was heterozygous for a 1 bp deletion – c.1886delT (p. Leu629Cysfs). This variant, on exon 15, causes a frameshift and it is classified as pathogenic. For that reason, this couple is eligible for PGT treatment.

A Multiplex-PCR was already available *in-house*, so this protocol was firstly tested for the couple. Primer design for the detection of the variant by minisequencing was performed. The first round of Multiplex-PCR with selected markers showed six informative markers (two upstream and four downstream). Segregation could not be established at this step since it was a *de novo* variant - only in the first PGT cycle it would be possible to establish segregation. The determination of the paternal haplotypes would allow us to determine the number of unique alleles on each haplotype but since no DNA samples of relevant family members were available, this was not possible. Among the four STRs downstream, only two were selected based on the number of unique alleles observed. The four selected markers, plus the amplification of the fragment that contains the variant in exon 15 (Table 10), were further tested at a DNA concentration of 100 pg / μ L. The reverse minisequencing primer was chosen for the detection of the variant. The results allowed to proceed to the validation step - four validation tests on 12 single lymphoblasts of the male partner and 12 blanks to assess ADO, contamination, and no amplification rates for each amplicon. One of the cells showed a monosomy and was not included in the calculation of these rates (Table 11). Amplification, ADO and contamination rates were within the limits set by our laboratory. The amplification rate was > 95 %, and ADO and contamination rates were < 5 % for all amplicons. This resulted in a 'green report' for the workup of this couple. This workup was classified as a 'Long-PCR' workup and took 18 weeks to be ready.

The first cycle was initiated four months later (female age at oocyte retrieval: 28 years old). Three embryos were biopsied and sent to the genetic unit in Brussels. The results of the first PGT cycle are detailed in Table 12. Diagnosis was given for each individual cell and combined to diagnose the embryo. During this cycle, segregation was established.

The segregation analysis showed that the allele that the couple shared for D5S2051 and APCstrAGAT was located on the mutant haplotype. All selected STR markers have a unique allele on the male WT haplotype, meaning that unaffected embryos should be

heterozygous for all STR markers. In this scenario, it is possible to easily detect ADO (ADO can only be detected in heterozygous markers).

Embryo 1 was diagnosed as unaffected and genetically suitable for transfer. One amplicon showed ADO in one blastomere, but the male WT allele was present, reinforcing the diagnosis. Embryo 2 was classified as abnormal, since blastomere number 2 showed amplification for 3 alleles for all STR markers (two from the mother, and the affected allele from the father). Blastomere number 1 was diagnosed as affected, but the final combined diagnosis of the embryo was abnormal due to a trisomy. Finally, embryo 3 was classified as affected – blastomere number 1 showed failed amplification, but blastomere number 2 allowed diagnosing this embryo. Since day 3 biopsy followed by targeting testing allows a fresh transfer, embryo 1 was transferred at day 5, but no pregnancy was achieved – negative bHCG.

Table 10. Chosen STR markers for the couple – the male is affected, and the female is unaffected. Segregation was not established due to a *de novo* variant. The pathogenic allele is indicated with the symbol (*).

Locus (location relative to the gene)	Unaffected female	Affected male
D5S2051 (1.06 Mb 5')	230 - 232	232 - 234
APCstrAGAT (941 Kb 5')	157 - 157	157 - 161
c.1886delT (amplicon APCex15)	A / A	A / G*
D5S346 (32 Kb 3')	109 - 111	113 - 121
APCstrACB (299 Kb 3')	225 - 219	221 - 223

Table 11. Validation of the Multiplex-PCR protocol. For each amplicon, amplification, ADO, and contamination rates are calculated. Pre-defined thresholds: over 95 % of amplification and less than 5 % of ADO and contamination.

Locus (location relative to the gene)	Amplification (%)	ADO (%)	Contamination (%)
D5S2051 (1.06 Mb 5')	47/47 (100 %)	47/47 (0.0 %)	0/48 (0.0 %)
APCstrAGAT (941 Kb 5')	47/47 (100 %)	47/47 (0.0 %)	0/48 (0.0 %)
APCex15	47/47 (100 %)	47/47 (0.0 %)	0/48 (0.0 %)
D5S346 (32 Kb 3')	47/47 (100 %)	47/47 (0.0 %)	0/48 (0.0 %)
APCstrACB (299 Kb 3')	47/47 (100 %)	47/47 (0.0 %)	0/48 (0.0 %)

Table 12. Embryo genotype results. During this first PGT cycle, segregation was established. Two blastomeres of three embryos were tested, two single blastomeres each. The allele located on the mutant haplotype is indicated with the symbol (*). ADO: allele dropout; UA: unaffected; Aff: affected; ND: no diagnosis.

Locus	Unaffected female	Affected male	Informativity	E1.1	E1.2	E2.1	E2.2	E3.1	E3.2
D5S2051	230-232	234- 232*	Partially informative	232-234	232-234	232- 232*	232-230- 232*		232- 232*
APCstrAGAT	157-157	161- 157*	Partially informative	157-161	157-161	157- 157*	157-157- 157*		157- 157*
c.1886delT	A / A	A / G*		A / A	A / A	A / G*	A / G*		A / G*
D5S346	109-111	113- 121*	Fully informative	ADO-113	111-113	111- 121*	109-111- 121*		111- 121*
APCstrACB	225-219	223- 221*	Fully informative	219-223	219-223	219- 221*	219-225- 221*		219- 221*
Diagnosis of the cell				UA	UA	Aff	Abnormal (trisomy)	ND (No amplification)	Aff
Diagnosis of the embryo				Unaffected		Abnormal (trisomy)		Affected	

A second cycle was initiated one year later (female age at oocyte retrieval: 29 years old). In this PGT cycle, 7 embryos were biopsied and sent to the genetic unit in Brussels. The results are detailed in Table 13.

Embryos 2, 3, 4 and 6 were diagnosed as unaffected and suitable for transfer. For embryos 2, 3, 5 and 6, ADO was detected. Blastomere number 1 of embryo 3 had an inconclusive diagnosis because, for the markers upstream of the variant, no amplification was observed for D5S2051 marker and only one allele amplified for APCstrAGAT marker – in this case, the allele that amplified was an allele shared between the couple, so it was not possible to distinguish its origin. Blastomere number 2 allowed to diagnose the embryo as all markers showed amplification, resulting in the combination of a maternal and paternal haplotype. The same situation was observed for embryo number 6. Embryos number 1 and 8 were diagnosed as abnormal, since one of the blastomeres only showed amplification of one allele for each STR marker, indicating the presence of only one paternal haplotype. Finally, embryo 5 was diagnosed as affected, even though one of the cells had an inconclusive diagnosis due to ADO.

After diagnosis, 2 embryos were transferred at day 5, but no pregnancy was achieved – negative bHCG. The other two genetically transferable embryos were cryopreserved. In 2018, another cycle was initiated - both cryopreserved embryos were thawed but were degenerated. Therefore, no transfer was performed.

Table 13. Embryo genotype results. Seven embryos were tested, two single blastomeres each. The allele located on the mutant haplotype is indicated with the symbol (*). ADO: allele dropout; UA: unaffected; Aff: affected; ND: no diagnosis.

Locus	Unaffected female	Affected male	Informativity	E1.1	E1.2	E2.1	E2.2	E3.1	E3.2
D5S2051	230-232	234- 232*	Partially informative	232-232*	232	232-234	232-234	ADO-ADO	230-234
APCstrAGAT	157-157	161- 157*	Partially informative	157-157*	157	157-161	157-161	157-ADO	157-161
c.1886delT	A / A	A / G*		A / G*	A	A / A	A / A	A / A	A / A
D5S346	109-111	113- 121*	Fully informative	111-121*	111	111-113	ADO-113	109-113	111-113
APCstrACB	225-219	223- 221*	Fully informative	219-221*	219	219-223	219-ADO	225-223	225-223
Diagnosis of the cell				Aff	Abnormal (monosomy)	UA	UA	ND - inconclusive	UA
Diagnosis of the embryo				Abnormal (monosomy)		Unaffected		Unaffected	

Locus	E4.1	E4.2	E5.1	E5.2	E6.1	E6.2	E8.1	E8.2
D5S2051	232-234	232-234	232-232*	232-232*	232-234	ADO-ADO		234
APCstrAGAT	157-161	157-161	157-157*	157-161-157*	157-161	157-ADO		161
c.1886delT	A / A	A / A	A / G*	A / ADO	A / A	A / A		A
D5S346	111-113	111-113	111-121*	111-121*	111-113	111-113		113
APCstrACB	219-223	219-223	219-221*	219-ADO	219-223	219-223		223
Diagnosis of the cell	UA	UA	Aff	ND - inconclusive	UA	ND - inconclusive	ND (No amplification)	Abnormal (monosomy)
Diagnosis of the embryo	Unaffected		Affected		Unaffected		Abnormal (monosomy)	

4.2 Family history, indications and workup

From January 2006 until February 2021, 284 couples requested PGT treatment for 120 different indications (for PGT-M, PGT-M combined with HLA matching and solely HLA matching) (Fig. 14). A family member was available for over 85 % of the couples. Generally, only one family member is available (80 % of the cases).

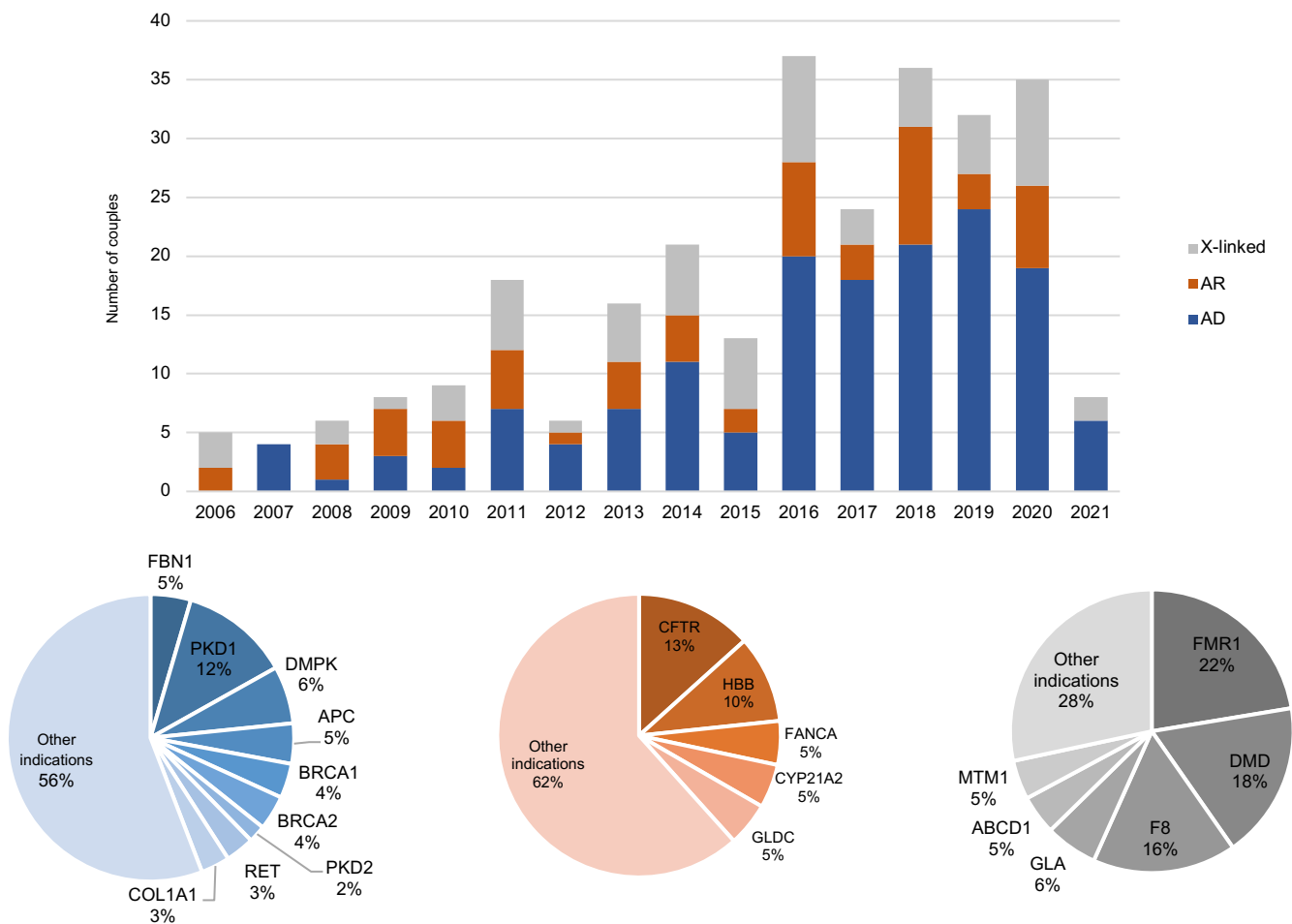


Figure 14 – PGT requests from January 2006 until February 2021. **Top:** evolution over the years. **Bottom:** Most common indications according to their mode of inheritance. In blue, the most common AD inherited disease is *PKD1*, polycystic kidney disease 1, followed by *DMPK*, myotonic dystrophy and *APC*, familial adenomatous polyposis. In orange, the most frequent AR diseases requested are *CFTR*, cystic fibrosis, *HBB*, sickle cell anemia and *FANCA*, Fanconi Anemia. Lastly, in grey, the most requested X-linked disorders are *FMR1*, fragile X syndrome, *DMD*, Duchenne muscular dystrophy and *F8*, hemophilia.

As mentioned before, variants were classified according to their pathogenicity (excluding the trinucleotide expansion variants and cases of HLA matching for an acquired disorder) (Fig. 15). As expected, most variants were classified as pathogenic or likely pathogenic, or presented a double classification as pathogenic/likely pathogenic. However, 13

variants belong to class 3 (unknown significance) and two variants present a conflicting interpretation of pathogenicity on public databases. A *de novo* variant occurred in 20 couples (7 % of the total requests). There were 14 couples requesting testing for HLA matching, 7 for acquired disorders and 7 for combination with PGT-M (Table 14). Although not all these couples proceeded to a clinical cycle, 9 cycles were initiated for HLA matching requests and 8 cycles for combined HLA and PGT-M requests (Fig. 13).

In the early years, Multiplex-PCR was the most widely used technique in PGT treatment. Until 2015, only PCR was applied and, for that reason, 72 % of the total number of workups performed (n = 271) were done with this technique. The Multiplex-PCR workup is divided in short-PCR (a protocol and/or primers were already available *in house*) and long-PCR (new primers and/or protocols had to be designed). SNP*a* was implemented more recently - it was done for the first time in transport PGT service for an HLA matching request in 2015 and for a second PGT workup in 2017. Since 2018, this technique started to be applied to more and more couples, representing most of the workups since 2019. Also, the test strategy has recently changed from PCR to SNP*a* for 11 couples (Fig. 16).

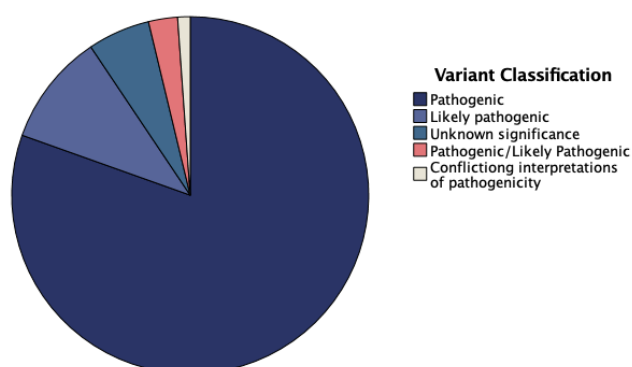


Figure 15 – Graphic representation of the variant classification (n = 271).

Table 14. Disease and OMIM number of diseases requested for PGT-M combined with HLA matching (number of couples = 7).

Disease	OMIM number disease	Frequency
Fanconi anaemia, complementation group A	227650	3
Thalassemia, beta	613985	2
Chronic granulomatous disease	306400	1
Treacher-collins Franceschetti Syndrome	154500	1

The Multiplex-PCR workups take up a lot of time, especially the long-PCR. The Turnaround Time (TAT) of the workup - the time interval from the moment all samples are available to the time of the report - is, on average, 19 weeks for a short-PCR and 53 weeks for a long-PCR. The SNPa workup is normally faster, but in cases with an insufficient number of key SNPs, it is necessary to add STR marker analysis and/or the mutation detection to the workup. For that reason, SNPa workup has an average TAT of about 16 weeks (Fig. 17).

The workup result is classified in 'green light' or 'red light'. A 'green light' report was given to 87 % of the requests (n = 248) while a 'red light' report was given to 8 couples (3 % of the requests) – seven PGT-M requests (facioscapulohumeral muscular dystrophy type 1, periventricular nodular heterotopia 1, two requests for Fabry disease, macular dystrophy vitelliform 2, neurofibromatosis type 1 and tuberous sclerosis 2) and one request for HLA matching (acute myeloid leukemia). For most of these requests, the workup was done with PCR, and it was not possible to establish segregation with STR markers. For the HLA matching request, a 'red light' report was given due to time constraints. 5 % of the couples (n = 14) cancelled the procedure due to several reasons, such as divorce (n = 3), financial problems (n = 1), lack of family members (n = 1), among others. The remaining 5 % are waiting for the result (data from 28th February 2021).

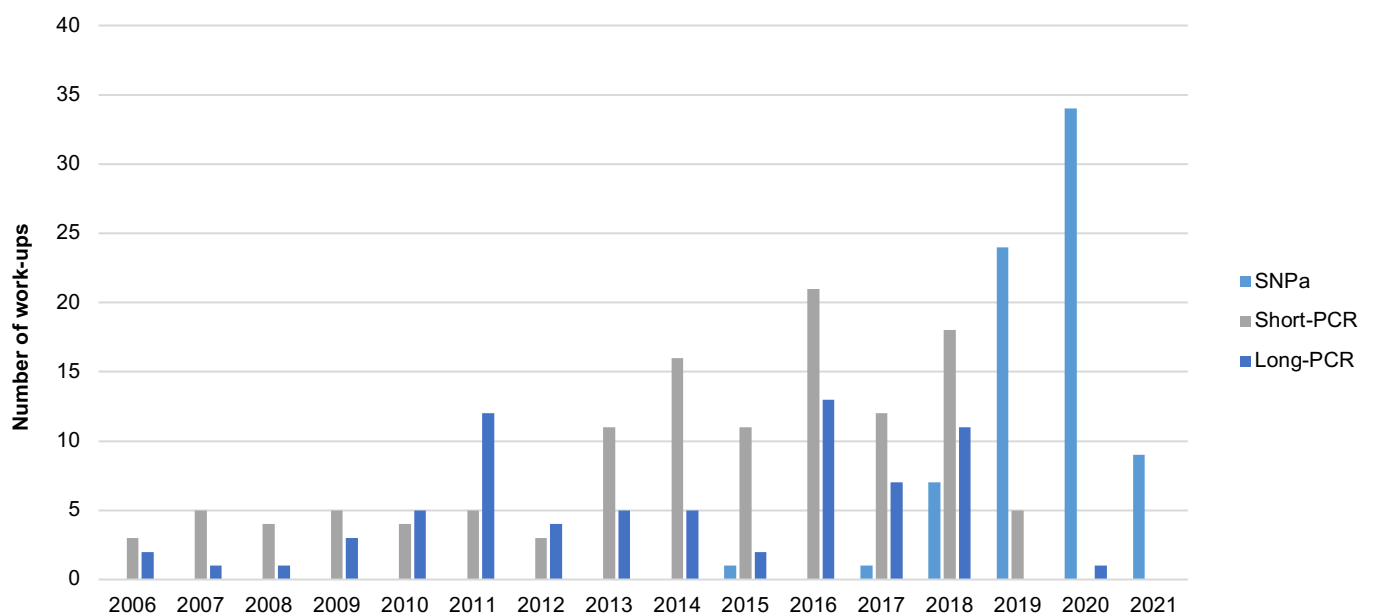


Figure 16 – Graphic representation of the number of workups performed with each technique over the years.

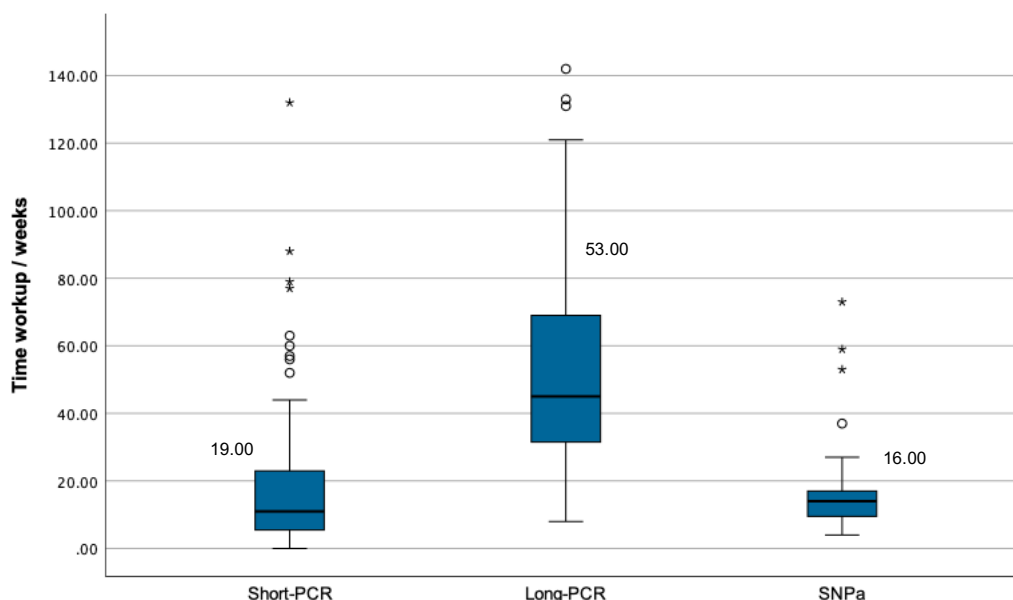


Figure 17 - Boxplots showing the TAT of the workup (weeks). Average time is detailed.

4.3 Clinical cycles

More than half of the couples that received a 'green light' report (68.1 %, n = 166) started a clinical cycle. Possible reasons for dropout after a 'green light' report are the long waiting period, spontaneous pregnancy, divorce, among others. As some couples had more than one clinical cycle started, 374 cycles were initiated. From these, 14 did not have an oocyte retrieval and 10 did not have an embryo biopsy. The clinical cycles were subdivided into cycles with a cryopreservation process before embryo transfer and cycles with a fresh biopsy, according to their initial purpose – PGT-M, HLA matching and PGT-M combined with HLA matching. The clinical outcome of each cycle was also reported (Table 15).

Table 15. Outcome of each cycle (oocyte freeze, embryo freeze at either day 2 or 3 (D2/3) of development, fresh embryo transfer (ET), no fresh embryo transfer (no ET), freeze all, frozen embryo transfer (FRET)), according to the initial purpose.

Purpose vs Outcome	Cycle with cryo before embryo biopsy (n = 69)		Cycle with fresh embryo biopsy (n = 281)				Total
	Freeze oocytes	Embryo freeze D2/3	Fresh ET	No ET	Freeze all	FRET	
PGT-M	7	62	129	45	31	59	333 (95.1 %)
HLA matching			4	1	3	1	9 (2.6 %)
PGT-M + HLA matching			5	3			8 (2.3 %)
Total	7 (2.0 %)	62 (17.7 %)	138 (39.4 %)	49 (14.0 %)	34 (9.7 %)	60 (17.1 %)	350

The frozen embryo transfers (FRET, n = 60) should be divided into several categories (Table 16). Due to cycles for accumulation of embryos, 189 embryos (from 62 cycles) were frozen at either day 2 or 3 of development. These were thawed in the following cycles, biopsied and possibly transferred. Similarly, 7 cycles of oocyte freeze occurred – oocytes were thawed in the next cycles, fertilized and the resulting embryos were biopsied and possibly transferred.

Not all cycles initiated had a subsequent transfer (Table 15) – 49 cycles had no embryo transfer, and 34 had a freeze all strategy. For the 49 cycles with no embryo transfer, 31 had no genetically transferable embryos, and 17 had transferable embryos but they were not transferred neither cryopreserved (possibly due to bad morphology). Only one cycle had embryos cryopreserved but they were not transferred in a deferred cycle. The freeze all strategy is used for all SNP_a cycles – all 21 cycles with a biopsy at day 5/6 of development applied this strategy, and 10 transfers were then performed in a deferred cycle. For PCR diagnosis strategy, 13 cycles had to apply a freeze all strategy.

Table 16. Description of the frozen embryo transfers (FRET) – FRET after PCR or SNP_a, FRET of an embryo that was cryopreserved before biopsy, FRET of an embryo that resulted from an oocyte freeze, a mixed transfer (one fresh embryo and one embryo that was cryopreserved after day 2 or 3 of development) and FRET after two cryopreservation processes.

FRET						Total
FRET after PCR	FRET after SNP _a	FRET after D2/3 freeze	FRET after oocyte freeze	Mixed transfer	FRET after freeze D2/3 + freeze D5	
28	10	10	1	8	3	60

4.3.1 From oocyte retrieval to embryo diagnosis

Within the 350 cycles initiated, 3276 oocytes were retrieved and 1856 were successfully fertilized, resulting in an average fertilization rate of 72.3 %. Further development allowed 1423 embryos to be tested, either with PCR or SNP_a techniques. Female age, at the moment of oocyte retrieval, varied from 23 to 43 years old, with a median of 34 years old. Spearman's correlation test shows a weak but significant correlation between the age of the female and the number of oocytes retrieved, number of oocytes that have undergone ICSI, number of fertilized oocytes and number of embryos biopsied (all $p < 0.001$) (Table 17).

Female age was stratified into four groups - group 1: ≤ 30 years; group 2: 31-35 years; group 3: 36-40; group 4: ≥ 40 years. It was observed that female age has a negative impact on the number of oocytes retrieved, number of oocytes that have undergone ICSI, number of oocytes fertilized, number of embryos biopsied and number of abnormal/aneuploid embryos (Table 18, Fig. 18). As Multiplex-PCR was firstly implemented, most embryos were biopsied at

day 3 (94 %) of development. In total, 1425 embryos were biopsied, and 1423 embryos were tested (Table 19).

Table 17. Correlations found between age of female, number of oocytes retrieved, number of oocytes that have undergone ICSI, number of fertilized oocytes and number of embryos biopsied. Strong* ($r > 0.60$) and very strong** ($r > 0.80$) correlations are flagged. All correlations shown are statistically significant ($p < 0.001$).

<i>r</i>	Age female	No. oocytes retrieved	No. oocytes undergone ICSI	No. fertilized oocytes	No. embryos biopsied
No. oocytes retrieved	-0.196		0.921 **	0.809 **	0.604 *
No. oocytes undergone ICSI	-0.239			0.889 **	0.687 *
No. fertilized oocytes	-0.206				0.791 *
No. embryos biopsied	-0.323				

Table 18. PGT cycle laboratory outcomes and embryo biopsy results for 350 cycles, according to the age of the female. Data are shown as median (interquartile range). Pairwise Independent-samples Kruskal-Wallis Test, statistically significant results when $p \leq 0.050$.

Group	≤ 30	31-35	36-40	≥ 41	Total
No. of cases (%)	57 (17.8 %)	139 (43.4 %)	120 (37.5 %)	4 (1.3 %)	320
Female age (years)	29.00 (2.00)	33.00 (2.00)	38.00 (2.00)	42.50 (1.00)	---
No. oocytes retrieved	12.50 (9.00) *	12.00 (8.00) *	9.00 (7.00)	7.50 (6.00)	3276
No. oocytes undergone ICSI	10.00 (6.00) *	10.00 (6.00) *	7.00 (4.00)	6.50 (4.00)	2566
No. fertilized oocytes	6.50 (5.00) *	7.00 (6.00) *	5.00 (3.00)	4.50 (4.00)	1856
No. embryos biopsied	5.00 (4.00) **	6.00 (5.00) **	4.00 (2.00)	3.00 (2.00)	1425
No. abnormal/aneuploid embryos	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	2.00 (4.00)	172

* $p \leq 0.050$ compared to the group age 36-40 years old

** $p \leq 0.050$ compared to the group age 36-40 years old and to the group age ≥ 41 years old

Embryos were classified as genetically transferable or genetically not transferable (Table 20). SNP_a has the advantage of giving a diagnosis for both PGT-M and PGT-A, allowing the transfer of only embryos not affected by the disease and euploid. PCR technique only explores the gene of interest and 1 Mb flanking regions – it is not possible to evaluate the status of other chromosomes. However, it is possible to detect aneuploidies in the chromosome of interest. For that reason, it is important to distinguish embryos that were not transferred due to a PGT-M diagnosis or due to chromosomal abnormalities (for PCR) or a

PGT-A diagnosis (for SNPa). A statistically significant difference was found between the percentage of genetically transferable embryos and the genetically not transferable embryos diagnosed by PCR or SNPa. Likewise, a statistically significant difference was found between the percentage of genetically not transferable embryos due to abnormalities/PGT-A diagnosis by PCR or SNPa (Table 20).

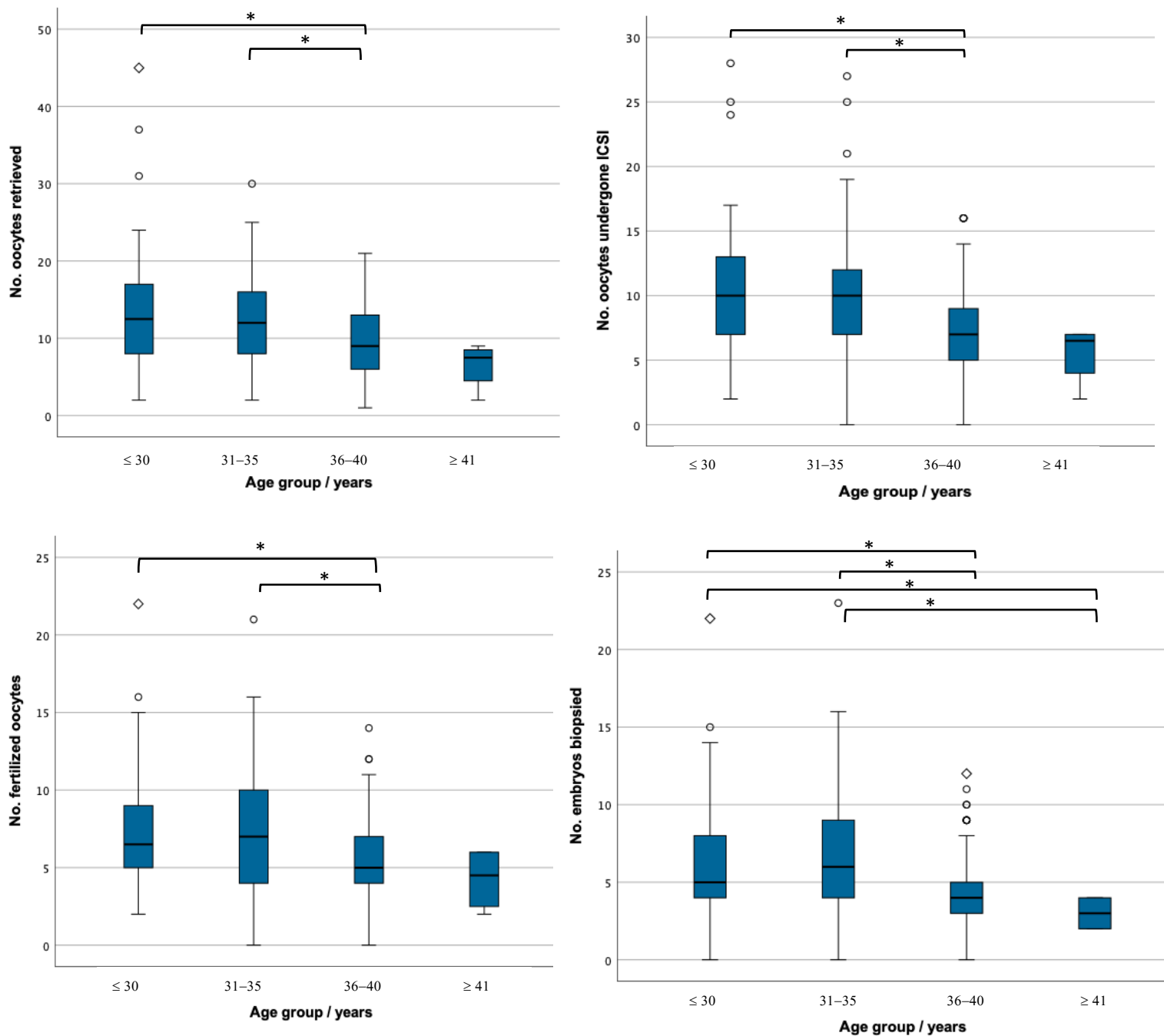


Figure 18 – Graphic representation of the distribution of the number of oocytes retrieved, number of oocytes that had undergone ICSI, number of fertilized oocytes and number of embryos biopsied according to the group age of the female. Pairwise independent-samples Kruskal-Wallis Test, statistically significant results when $p \leq 0.050$.

Table 19. Number of embryos tested and biopsied according to the day of the biopsy/technique used in the testing strategy.

Day of biopsy / Technique	Testing	Total	Median (Interquartile range)
Day 3 / Multiplex-PCR	Number of embryos biopsied	1336	5.00 (4)
	Number of embryos tested	1334	5.00 (4)
Day 5 / SNP _a	Number of embryos biopsied	89	4.00 (3)
	Number of embryos tested	89	4.00 (3)

A total of 140 embryos (138 for PCR and 2 for SNP_a) remained with no diagnosis (Table 20) due to several reasons such as ADO, contamination, recombination, or no amplification. There is a statistically significant difference among the percentage of embryos with no diagnosis between the two testing approaches ($p = 0.006$). Although the percentage of embryos with no diagnosis due to ADO, contamination, or recombination in the two approaches was not statistically different, the percentage of embryos with no diagnosis due to no amplification was significantly different between Multiplex-PCR and SNP_a ($p = 0.012$) (Fig. 19).

Table 20. Number of genetically transferable and genetically not transferable embryos according to the day of the biopsy/technique used in the testing strategy. For both strategies, the number of genetically not transferable embryos due to PGT-M diagnosis and due to chromosomal abnormalities/PGT-A diagnosis is distinguished. Percentage of embryos with each diagnosis was compared between both testing strategies. Independent-samples Mann-Whitney U Test, statistically significant results when $p \leq 0.050$.

Diagnosis	Number of embryos (Percentage)		p-value
	Day 3 / Multiplex-PCR	Day 5 / SNP _a	
Genetically transferable	519 (38.9%)	17 (19.1%)	0.018
Genetically not transferable	815 (61.1%)	72 (79.8%)	0.003
Genetically not transferable due to PGT-M	529 (64.9%)	46 (63.9%)	0.103
Genetically not transferable due to chromosomal abnormalities	148 (18.2%)	24 (33.3%)	0.001
Genetically not transferable due to no diagnosis	138 (17.0%)	2 (2.78%)	0.006

The destiny of the embryos is considered after obtaining a diagnosis. For instance, for a day 3 biopsy, a cycle can be initiated, and one or two embryos can be transferred in the same cycle. If more embryos are diagnosed as genetically suitable for transfer, there is the possibility of cryopreservation and transfer in a deferred cycle. For day 5/6 biopsy, all embryos are cryopreserved, in the so-called ‘freeze all’ strategy while waiting for diagnosis.

A total of 423 embryos were cryopreserved – all 89 embryos biopsied at day 5/6 and 334 embryos before or after a day 3 biopsy. Also, 28 oocytes were cryopreserved. Cryopreservation method was, since 2008, the vitrification method.

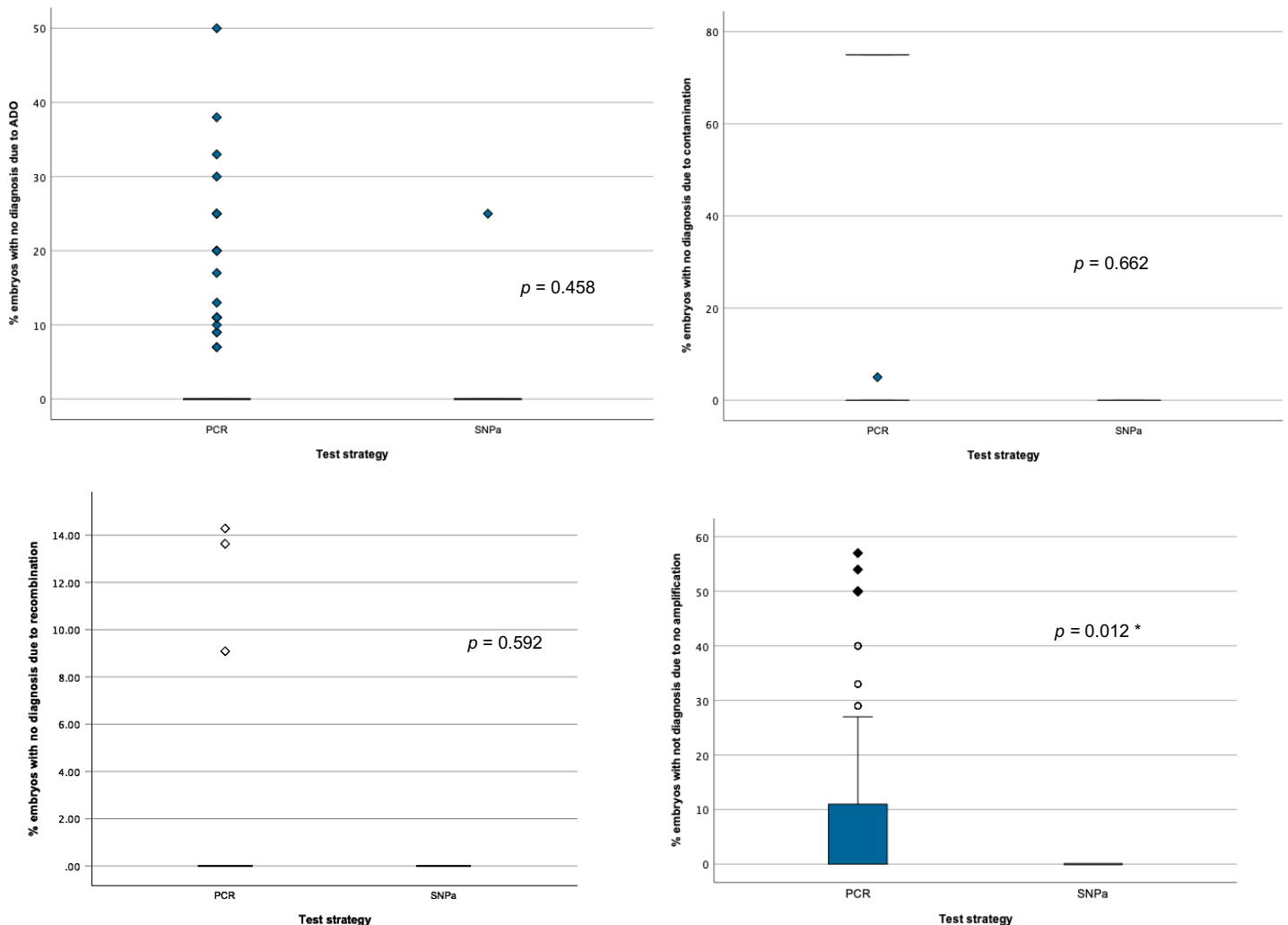


Figure 19 – Graphic representation of the distribution of the percentage of embryos with no diagnosis due to ADO, contamination, recombination, and no amplification for each testing approach (Multiplex-PCR or SNPα). Independent-samples Mann-Whitney U Test, statistically significant results when $p \leq 0.050$.

4.3.2 Embryo transfer and pregnancies

From the 536 genetically suitable embryos, 297 embryos were transferred over 200 transfer cycles (Table 21). A positive bHCG was achieved in 65 of the transfers (32.5 %). The overall implantation rate, calculated as observed gestational sacs per total number of embryos transferred, was 16.8 %; the overall clinical pregnancy rate per embryo transfer (CPR) was 24.5 %. The live birth delivery rate per embryo transfer (LBDR) was 21.0 %. Chi-square analysis was run to assess differences between the types of transfer and specific outcomes (pregnancy, no pregnancy, biochemical pregnancy, miscarriage, EUG, stillbirth, and ongoing

pregnancy) but did not show a statistically significant difference ($p = 0.302$), meaning that the observed distribution is not different from the expected distribution. If we only consider the ‘traditional pathways’ of PGT and compare the implantation rate and CPR (Table 21), it is possible to observe a higher rate in both parameters when embryos are biopsied at day 5.

Table 21. Descriptive data of the number of embryos transfers in each category, both fresh and frozen. Number of transfers that had a positive bHCG, number of clinical pregnancies, observed gestational sacs, average implantation and pregnancy rates, and pregnancy outcomes - biochemical pregnancy, miscarriage, extra-uterine gestation (EUG), stillbirth (death after 20 weeks) and term pregnancies. NA – not applicable.

	Type of transfer							Total
	Fresh ET	FRET after PCR	FRET after SNP _a	FRET after D2/3 freeze	FRET after oocyte freeze	Mixed transfer	FRET after freeze D2/3 + freeze D5	
No. single embryo transfers	72	16	10	3	1	0	2	104
No. multiple embryo transfer (2 embryos)	68	12	0	7	0	8 ^{a)}	1	96
Total of embryos transferred / total of embryo transfers (ET)	208 /140	40 / 28	10 / 10	17 / 10	1 / 1	17 / 8	4 / 3	297 /200
No. positive bHCG	42	9	5	4	0	4	1	65
No. of clinical pregnancies	33	6	3	3	0	4	0	49
No. gestational sacs observed	32	6	4	4	0	4	0	50
Average implantation rate	15.4 %	15.0 %	40 %	23.5 %	NA	23.5 %	0.0 %	16.8 %
Clinical pregnancy rate per embryo transfer (CPR)	23.6 %	21.4 %	30.0 %	30.0 %	NA	50.0 %	0.0 %	24.5 %
No. biochemical pregnancies	9	3	1	0	--	0	1	14
No. miscarriages	3	1	0	0	--	1	0	5
No. EUG	1	0	0	0	--	0	0	1
No. stillbirth	1	0	0	0	--	0	0	1
No. ongoing	0	0	1	1	--	0	0	2
Term pregnancies (number of babies)	28 (31)	5 (6)	3 (3)	3 (3)	NA	3 (4)	0	42 (47)
Live birth delivery rate per embryo transfer (LBDR) (%)	20.0 %	17.8 %	30 %	30.0 %	NA	37.5 %	0.0 %	21.0 %

^{a)} From these 8 transfers, 17 embryos were transferred – 9 fresh and 8 frozen at either day 2 or 3 of development. One transfer had 3 embryos transferred, 2 fresh and 1 frozen.

From the 65 positive bHCG, 42 pregnancies were achieved. Two pregnancies (one FRET after SNPa and another after FRET after D2 freeze) are still ongoing. The 42 pregnancies achieved (37 singleton and five twin pregnancies) were distributed over 39 couples, all with PGT-M requests. For four couples, 8 cycles were initiated combining PGT-M with HLA matching which resulted in five transfers, four with a negative bHCG and one with a biochemical pregnancy. For HLA matching requests, one couple initiated 8 cycles, one with no oocyte retrieval, one with no embryo biopsy, one with no transfer and 5 with embryo transfers that resulted in 4 negative bHCG and 1 biochemical pregnancy.

Considering the couples that achieved a pregnancy, only 2 couples performed a pre-natal test (one had a chorion villus sampling and the other an amniocentesis) done to confirm the PGT diagnosis, both couples with a PGT-M request, one for fragile X syndrome and the other one for mucopolidosis II alpha/beta.

4.3.3 Term & newborn characteristics

A term pregnancy is considered after 37 weeks of gestation. Pregnancies were classified as term, late preterm and moderate preterm (Table 22, Fig. 20). Similarly, the birth weight was classified as normal, low birth weight (LBW) and very low birth weight (VLBW) (Table 23, Fig. 21). There is a statistically significant difference for both parameters between singleton and twin newborns. Of the five twin pregnancies, two pregnancies had one baby with normal birth weight and one baby with LBW. In the other three, both babies had the same classification for this parameter.

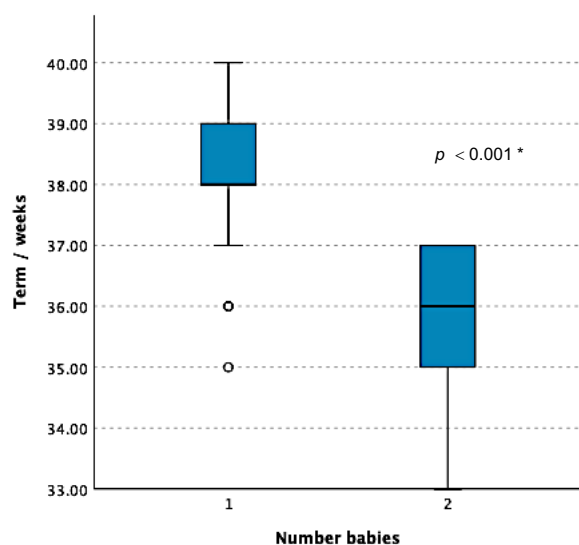


Figure 20 – Boxplot of the distribution of the gestational age (weeks) for singleton and twin pregnancies. Independent-samples Mann-Whitney U Test, statistically significant results when $p \leq 0.050$.

Table 22. Gestational age of the achieved pregnancies.

Gestational age	Singleton pregnancies	Twin pregnancies
Term (≥ 37 weeks)	28	0
Late preterm (34 to < 37 weeks)	9	4
Moderate preterm (32 to < 34 weeks)	0	1

Table 23. Birth weight of the newborn children. LBW: low birth weight; VLBW: very low birth weight.

Birth weight	Singleton babies	Twin babies
Normal (≥ 2500 g)	35	4
LBW (< 2500 g)	2	6
VLBW (< 1500 g)	0	0

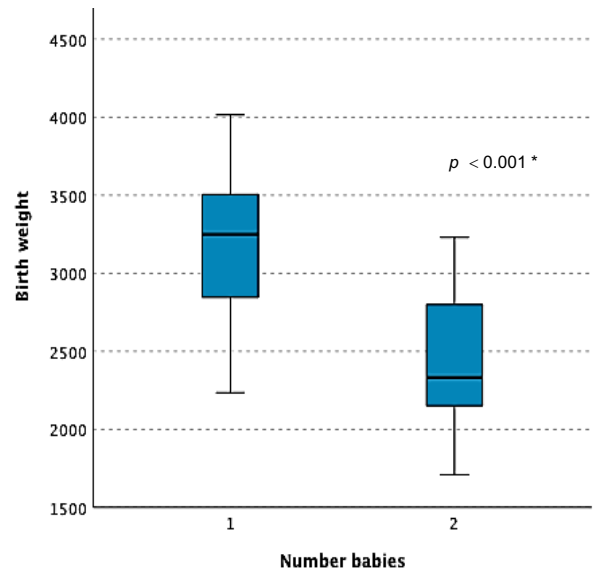


Figure 21 – Boxplot of the distribution of the birth weight (grams) for singleton and twin pregnancies. Independent-samples Mann-Whitney U Test, statistically significant results when $p \leq 0.050$.

If we consider only the singleton pregnancies, no statistically significant differences were found between the type of transfer and both the term of pregnancy and the birthweight (Fig. 22). Considering only the ‘traditional pathways’, these comparisons did not show a statistically significant difference (Fig. 23).

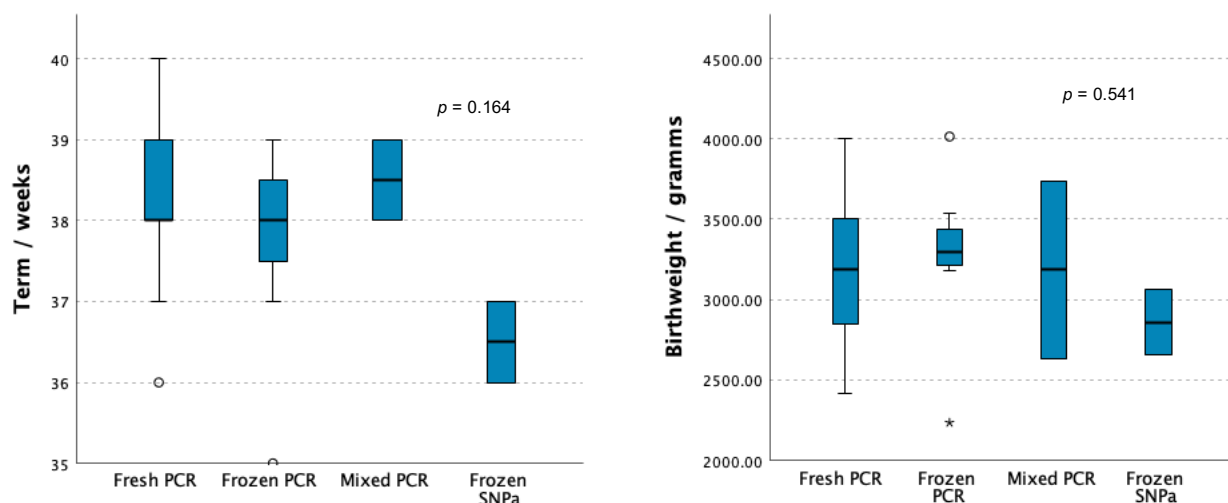


Figure 22 – Boxplot of the distribution of the term (weeks) of pregnancy and birthweight (grams) according to the test strategy and type of transfer. Only singleton pregnancies were compared. Independent-samples Kruskal-Wallis Test, statistically significant results when $p \leq 0.050$.

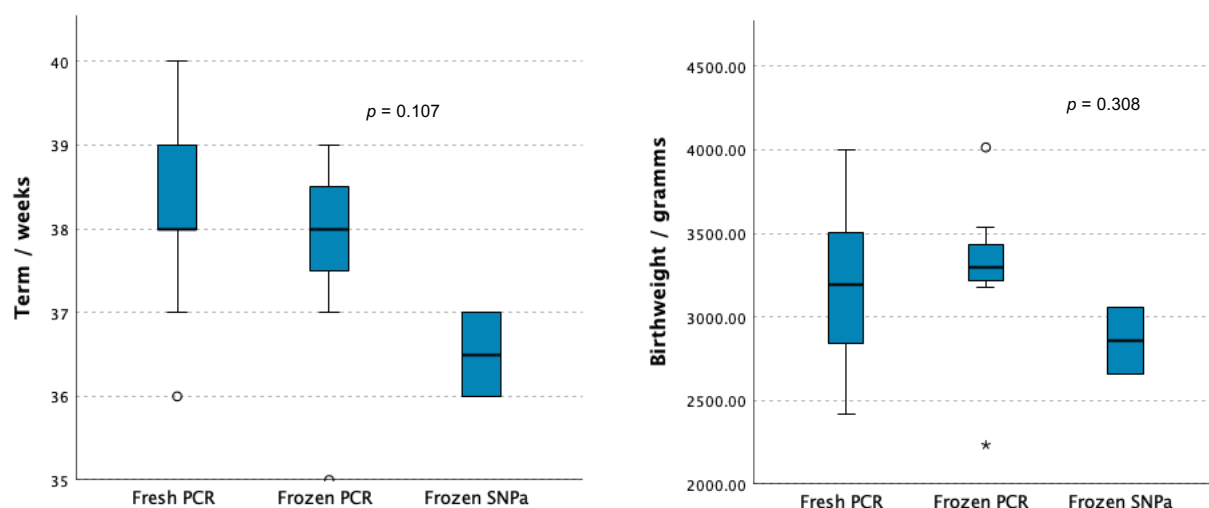


Figure 23 – Boxplot of the distribution of the term (weeks) of pregnancy and birthweight (grams) according to the day of biopsy and test strategy. Only singleton pregnancies were compared. Independent-samples Kruskal-Wallis Test, statistically significant results when $p \leq 0.050$.

4.4 In-house comparisons

Comparisons between transport cycles and *in-house* Brussels cycles were performed for a smaller set of data – from 2015 to 2019. Both 2-blastomere biopsy and TE biopsy cycles were included. Clinical cycles with a cryopreservation process before embryo transfer ($n = 54$) were excluded from the analysis since there is only a contribution from transport PGT cycles and not *in-house* cycles. Cycles were grouped according to their initial purpose – PGT-M, HLA matching and PGT-M combined with HLA matching - and their clinical outcome (Table 24). A total of 1767 cycles were initiated – 16 had no oocyte retrieval (7 in transport and 9 *in-house*), 107 had no biopsy (7 in transport and 100 *in-house*) and 189 *in-house* cycles had a ‘freeze all, no cryo’ outcome. A frozen transfer was performed in 689 cycles – 146 cycles after a day 3 biopsy and cryopreservation at day 5 and 537 cycles after a day 5/6 biopsy and SNPa.

Females from transport cycles ($n = 240$) had a median age of 34 years old, while females from *in-house* cycles ($n = 912$) had a median of 32 years old ($p < 0.001$).

Within the subset of 1455 cycles initiated (252 transport PGT cycles and 1263 *in-house* cycles), 12558 oocytes were retrieved and 7777 were successfully fertilized resulting in an average fertilization rate of 62.0 % (56.2 % transport PGT and 63.3 % *in-house* cycles), which is lower than the one obtained for the transport cycles alone. Further development allowed 4502 embryos to be tested (1097 transport PGT and 3405 *in-house*), either with PCR ($n = 2228$) or SNPa ($n = 2271$) techniques.

Table 24. Outcome of each cycle (fresh embryo transfer (ET), no fresh embryo transfer (no ET), freeze all, frozen embryo transfer (FRET)), according to the initial purpose. Number of cycles, between 2015 and 2019, are detailed as Porto + Brussels.

Purpose vs Outcome	Cycle with fresh embryo biopsy				Total
	Fresh ET	No ET	Freeze all	FRET	
PGT-M	98 + 82	33 + 41	19 + 429	31 + 658	702 (150 + 552)
HLA matching	4 + 0	1 + 0	3 + 2		10 (8 + 2)
PGT-M + HLA matching	0 + 2	0 + 4	3 + 45		54 (3 + 51)
Total	186 (12.78 %)	79 (5.43 %)	501 (34.43 %)	689 (47.35 %)	1455 (192 + 1263)

Embryo diagnosis was assessed as done previously (Table 25). As predicted, a statistically significant difference was found between the two testing strategies and several parameters, such as the percentage of embryos not suitable for transfer due to abnormalities/PGT-A, that is higher for the SNP_a, and due to no diagnosis, which is higher for the PCR strategy.

Table 25. Number of genetically transferable and genetically not transferable embryos according to the day of the biopsy/technique used in the testing strategy. For both strategies, the number of genetically not transferable embryos due to PGT-M diagnosis and due to chromosomal abnormalities/PGT-A diagnosis is distinguished. Data from 2015 to 2019: numbers are detailed as Porto + Brussels. Percentage of embryos with each diagnosis was compared between both testing strategies. Independent-samples Mann-Whitney U Test, statistically significant results when $p \leq 0.050$.

	Number of embryos (Porto + Brussels)				p-value
Diagnosis	Day 3 / Multiplex-PCR		Day 5 / SNP _a		
Genetically transferable	897 (402 + 495)	40.26 %	902 (13 + 890)	39.67 %	0.079
Genetically not transferable	1331 (645 + 686)	59.74 %	1372 (39 + 1333)	60.33 %	0.148
Genetically not transferable due to PGT-M	924 (411 + 513)	69.42 %	913 (25 + 888)	66.54 %	0.162
Genetically not transferable due to chromosomal abnormalities	236 (121 + 115)	17.73 %	424 (14 + 410)	30.90 %	≤ 0.001
Genetically not transferable due to no diagnosis	171 (107 + 64)	12.85 %	35 (0 + 35)	2.55 %	≤ 0.001

A total of 206 embryos remained with no diagnosis. To assess if these differences could also be influenced by the transportation procedure, and not only by the test strategy, the percentage of embryos with no diagnosis and the possible reasons why they did not have a diagnosis were compared between the transport and the *in-house* cycles (Table 26). A day 3

biopsy followed by PCR testing shows a statistically significant difference between the percentage of embryos with no diagnosis between transport and *in-house* cycles ($p < 0.001$). The two major reasons that explain this difference is the ADO and the no amplification rates, both higher for transport cycles (Fig. 24). Day 5/6 biopsy followed by SNP_a did not show a statistically significant difference between transport and *in-house* cycles.

After the diagnosis report is ready, embryos can be transferred. From the 1799 genetically suitable embryos, 940 embryos were transferred over 862 transfer cycles (Table 27). A positive bHCG was achieved in 485 of the transfers (56.3 %). Overall, day 5/6 biopsy followed by SNP_a seems to have better PGT outcomes, due to a higher implantation rate, CPR and LBDR. Chi-square analysis was run to assess differences between the types of transfer and specific outcomes (pregnancy, no pregnancy, biochemical pregnancy, miscarriage, EUG, stillbirth, elective termination, and ongoing pregnancy) but failed to reach statistical significance ($p = 0.058$), meaning that the observed distribution is not different than the expected distribution.

In total, 19 pregnancies are still ongoing – the starting date of the cycle is 2019 but the transfers were only done in 2020. Considering the couples that achieved a pregnancy, 140 pregnancies asked for a pre-natal test. Concerning the post-natal analysis, only the term of the pregnancy was assessed - no statistically significant differences were found among the different types of transfer (Fig. 25).

Table 26. Transport and *in-house* PGT cycles comparisons on the percentage of embryos with each possible diagnosis outcome. Independent-samples Mann-Whitney U Test, statistically significant results when $p \leq 0.050$.

Day of biopsy / Technique	Percentage of embryos	p-value
Day 3 / Multiplex-PCR	Genetically transferable	0.208
	Genetically not transferable	0.147
	Due to PGT-M	0.128
	Due to abnormalities	0.072
	Due to no diagnosis	< 0.001
	- ADO - Contamination - No amplification - Recombination	< 0.001 0.095 < 0.001 0.226
Day 5 / SNP _a	Genetically transferable	0.063
	Genetically not transferable	0.051
	Due to PGT-M	0.127
	Due to PGT-A	0.126
	Due to no diagnosis	0.439
	- ADO - Contamination - No amplification - Recombination	1.000 0.896 0.503 0.793

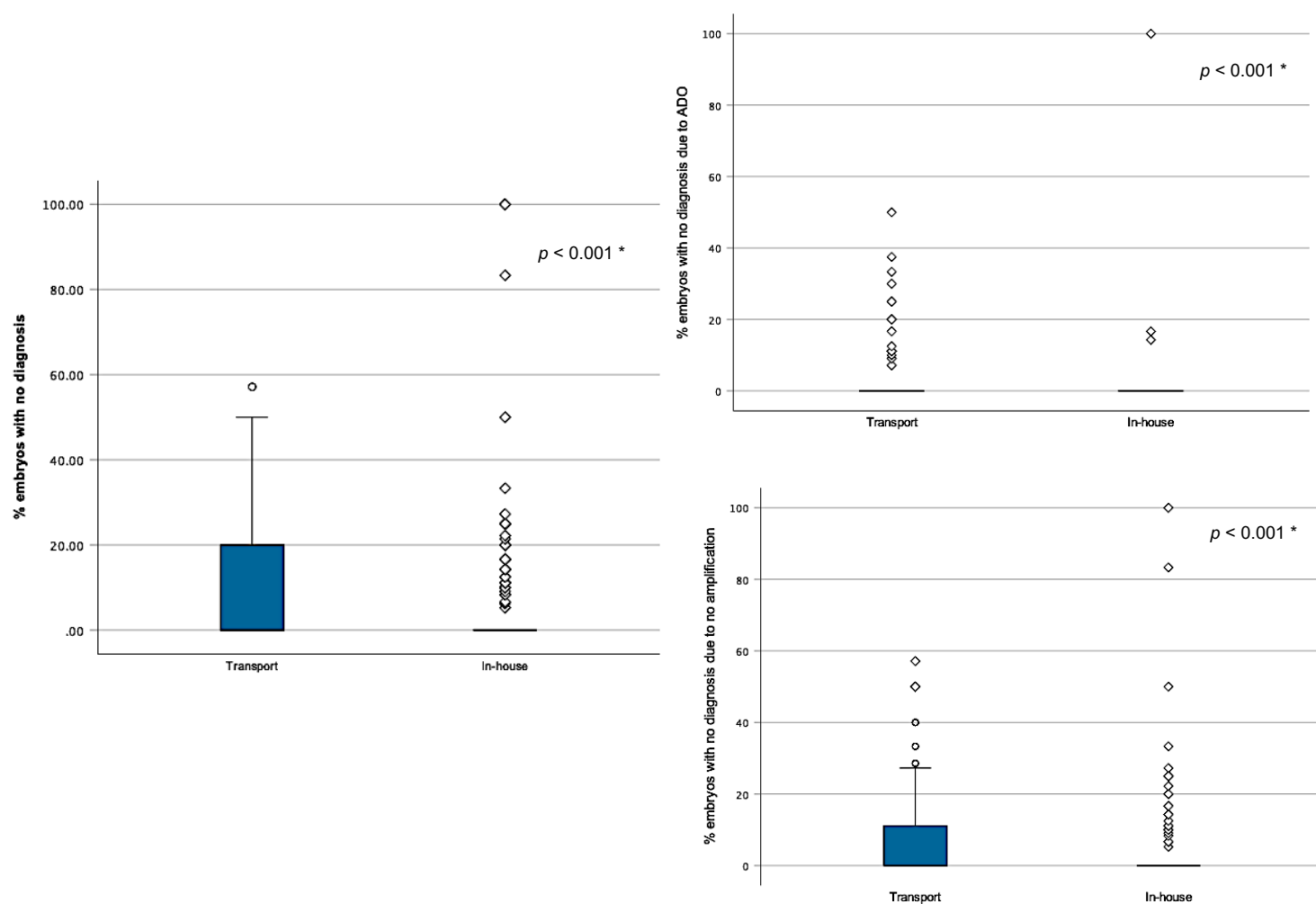


Figure 24 – Graphic representation of the distribution of the percentage of embryos with no diagnosis, and the percentage of embryos with no diagnosis due to ADO and no amplification between transport and *in-house* cycles, after a day 3 biopsy followed by targeted testing with PCR. Independent-samples Mann-Whitney U Test, statistically significant results when $p \leq 0.050$.

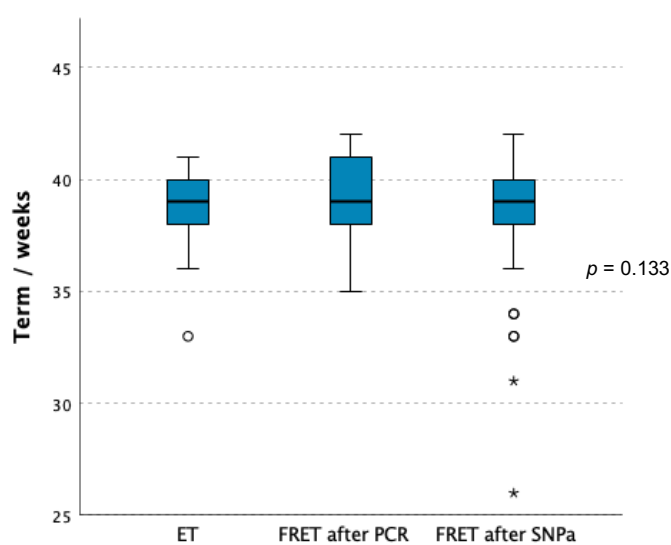


Figure 25 – Boxplot of the distribution of the term (weeks) of singleton deliveries according to the type of transfer. Independent-samples Kruskal-Wallis Test, statistically significant results when $p \leq 0.050$.

Table 27. Descriptive data of the number of cycles to oocyte retrieval, cycles to PGT, embryos transferred, transfers, positive bHCG, clinical pregnancies, observed gestational sacs, average implantation, clinical pregnancy, and live birth delivery rates among the 'traditional pathways' – fresh transfer after day 3 biopsy and PCR, frozen transfer (cryopreservation at day 5) after day 3 biopsy and PCR and frozen transfer after a day 5/6 biopsy and SNP. Data from 2015 to 2019 is detailed as Porto + Brussels.

	Fresh transfer after a day 3 biopsy / PCR		Frozen transfer after a day 3 biopsy / PCR		Transfer after a day 5 biopsy / SNP		Total
	Porto	Brussels	Porto	Brussels	Porto	Brussels	
No. cycles to oocyte retrieval and to PGT	97 (Porto) + 86 (Brussels)				10	690	883
Total of embryos transferred / total of embryo transfers (ET)	152 / 107	85 / 76	24 / 33	142 / 119	7 / 7	530 / 520	940 / 862
No. positive bHCG (% per ET)	37 (34.5 %)	35 (46.1 %)	7 (21.2 %)	63 (52.9 %)	3 (42.9 %)	340 (65.4 %)	485 (56.3 %)
No. gestational sacs observed	30	32	7	60	3	313	445
No. of clinical pregnancies	21	29	4	43	3	240	341
Average implantation rate	19.7 %	37.6 %	29.2 %	42.3 %	42.9 %	59.0 %	47.3 %
Clinical pregnancy rate per embryo transfer (CPR)	19.6 %	38.1 %	12.1 %	36.1 %	42.9 %	46.2 %	39.56 %
Term pregnancies (number of babies)	20 (22)	28 (29)	3 (4)	32 (38)	3 (3)	195 (215)	279 (315)
Live birth delivery rate per embryo transfer (LBDR) (%)	19.7 %	36.8 %	9.1 %	26.9 %	42.9 %	37.5 %	32.37 %
No. biochemical pregnancies	3	5	1	6	0	20	35
No. miscarriage	3	7	1	12	0	51	74
No. EUG	1	1	0	2	0	2	6
No. stillbirth	1	0	0	0	0	2	3
No. elective termination	0	0	0	0	0	4	4
No. ongoing	0	0	0	1	0	18	19

5. Discussion

The present study aimed to analyze, retrospectively, PGT-M transport cycles from Portuguese couples since January 2006 in several aspects. Differences between day 3 biopsy/targeted testing with day 5/6 biopsy/genome-wide testing methods were explored and compared with the outcomes of the transfers and pregnancies achieved.

5.1 Family history, indications and workup

The Genetics Department in Porto performs *in-house* cycles for certain PGT-M indications - for AD disorders: familial amyloidotic polyneuropathy, Huntington disease, Machado-Joseph disease, and myotonic dystrophy; for AR disorders: spinal muscular atrophy; and for X-linked disorders: hemophilia A. PGT-A and PGT-SR indications are also performed *in-house*. Other PGT-M indications are done in transport PGT.

More than half of the transport PGT-M indications were autosomal dominant (AD) diseases, followed by 22 % of autosomal recessive (AR) indications (Fig. 14). This is in line with data of the ESHRE PGT Consortium (64 % AD, 21 % AR and 15 % X-linked) (unpublished data for 2016 - 2017). The most common AD indications for these patients were polycystic kidney disease, type 1, which sums 12 % of the AD indications, followed by myotonic dystrophy 1, familial adenomatous polyposis type 1, and Marfan syndrome. For AR disorders, cystic fibrosis was the most common indication (13 %), followed by beta thalassemia, Fanconi anemia, adrenal hyperplasia due to 21-hydroxylase deficiency and glycine encephalopathy. For the X-linked disorders, fragile X syndrome was the most requested indication (22 %), followed by Duchenne muscular dystrophy, hemophilia A, Fabry disease, myotubular myopathy and adrenoleukodystrophy. Most of these indications are also reported by ESHRE as the most frequent indications worldwide [101]. Surprisingly, polycystic kidney disease 1 is not reported as one of the most frequent indications.

Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited renal disease, with an estimated prevalence of 3.29 in 10 000 in Europe [102]. Pathogenic variants in *PKD1* are the most common (approximately 78 % of the affected individuals) and are associated with more severe phenotypes than variants in *PKD2*, which are present in approximately 15 % of the affected individuals [103]. In the south of Portugal, more precisely in Alentejo, the last estimated prevalence of ADPKD was 1:2564 (3.90 in 10 000, in a population of 543442 inhabitants) [104], which is comparable to the European prevalence, hence not explaining the higher number of requests. Furthermore, there may be other factors underlying the increased number of PGT requests for this disease in the Portuguese population, like a greater awareness of the referring physicians and the affected individuals about this reproductive possibility.

Information about the variant(s) and their pathogenicity was collected for each couple. Since 2015, couples are only eligible for PGT treatment if they carry or are affected with variants of classes 4 or 5 [23, 76]. As expected, most variants were classified as pathogenic or likely pathogenic; some presented a double classification as pathogenic/likely pathogenic. However, 13 variants belong to class 3 of classification (unknown significance) and two present a conflicting interpretation of pathogenicity on public databases (Fig. 15). For these 15 cases, four had the request before 2015, meaning that the eligibility criteria were not in place yet. The remaining nine cases were still accepted for PGT – the CNPMA consortium had authorized PGT treatment for these patients, since there was strong evidence to suspect that the variant under study was indeed the cause of the disease.

The workups done with PCR take longer to be ready, and in case of the long-PCR this is linked with the need of designing new primers for STR markers and/or the detection of the variant, and especially with the necessity of protocol validation. On average, SNP_a workups are done faster comparing to short-PCR (average TAT of 16 weeks for SNP_a compared with 19 weeks for short-PCR). However, it may also take longer when there are not enough key SNPs present on the WT haplotype. In those situations, it is necessary to add to the test strategy STR markers and/or the detection of the variant – when primers are not available *in-house*, it is necessary to design them, which can also delay the workup report (Fig. 17).

5.2 Clinical cycles

More than half of the couples that had a ‘green light’ report started a clinical cycle, with different purposes – PGT-M, HLA matching or a combined request. Cycles were subdivided in two categories – with cryopreservation before embryo biopsy and with fresh embryo biopsy (Table 15). Cycles with cryopreservation before embryo biopsy correspond to those with cryopreservation of either oocytes or embryos at day 2 or 3 of development (cycles for accumulation of embryos). Not all cycles had a fresh transfer after diagnosis. For that reason, frozen transfers also had to be subdivided into several categories depending on the timing of the embryo cryopreservation (Table 16) – SNP_a forces a ‘freeze-all’ strategy after embryo biopsy, typically at day 5/6 of development, while the genetic testing results are pending. Embryos can also be cryopreserved at day 5/6 of development, after a day 3 biopsy. For transport cycles, there were 60 cycles for accumulation of embryos, meaning that embryo cryopreservation occurred before biopsy – however, if these embryos are transferred in a deferred cycle, it is necessary to consider the fact that these have undergone a cryopreservation process, by vitrification. The same applies for the cycles in which oocytes were cryopreserved before biopsy. In total, 8 cycles were categorized as ‘mixed transfer’ (one fresh embryo and one frozen embryo, cryopreserved at day 2 or 3 of development, were

transferred together). Lastly, three embryos were transferred after two cryopreservation procedures – before and after biopsy.

5.2.1 From oocyte retrieval to embryo diagnosis

Typical data of ART cycles was collected for all couples. A weak negative correlation, yet significant ($p < 0.001$), is observed between both the number of oocytes retrieved, the number of oocytes with enough quality to undergo ICSI, the number of fertilized oocytes and the number of embryos biopsied with the age of the female (Table 17). It is clear that younger women have more oocytes retrieved (Table 18, Fig. 18), and therefore it can be expected that they have a greater chance for a higher number of mature and fertilized oocytes, which can result in a higher number of embryos to be biopsied. It has been reported that older women are less responsive to gonadotropins, which results in a lower number of developing follicles and, consequently, a lower number of oocytes retrieved [105]. The statistical analysis showed that the number of oocytes retrieved, the number of mature oocytes (with enough quality to undergo fertilization by ICSI) and the number of fertilized oocytes is significantly different between the age group of 36-40 years and the groups of under 30 years old and 31-35 years old (Table 18), with a clear decrease as female age increase (Fig. 18).

Embryo diagnosis can be performed by two distinct techniques – a targeted approach with Multiplex-PCR or a genome-wide approach with SNP_a. A statistically significant difference was observed for the number of genetically transferable embryos analyzed with PCR and SNP_a (Table 20). SNP_a has a lower percentage of genetically transferable embryos due to a higher percentage of embryos with aneuploidies detected with SNP_a (33.3 %) rather than with PCR (18.2 %) ($p = 0.001$). Detection of aneuploidies is one of the major advantages of SNP_a. Multiplex-PCR has a less extensive view of the genome – the STR markers are chosen within 1 Mb from the gene of interest, which allows the detection of aneuploidies only for that region/chromosome. In contrast, SNP_a enables the detection of aneuploidies across the entire genome. In addition, SNP_a is also capable of detecting unbalanced translocations, partial aneuploidies, triploidy and uniparental disomy, as well as chromosomal mosaicism [106].

It has been suggested that women's natural fertility follows an inverse U-curve with limited fertility for young and advanced female age, determined by aneuploidy rates in oocytes. That same paper also reported that aneuploid embryos originate from distinct age-dependent meiotic errors in oocytes [107]. Our data showed a higher number of abnormal/aneuploid embryos in females over 41 years old (Table 18), even though no statistically significant results were found. Embryo aneuploidy is an important factor since it is one of the major constraints of human reproduction and might lead to implantation failure, miscarriage and congenital malformations [108, 109]. PGT-A is considered a screening test, usually carried out for a

specific IVF patient group with a low genetic risk, such as advanced maternal age, recurrent miscarriages, where it is expected that selection against aneuploid embryos will improve the IVF outcome and decrease the miscarriage rates. SNP_a, being a genome-wide technology, allows concurrent PGT-M and PGT-A. The concurrent testing does not involve extra risks for the couple or the future child and the laboratory workload is usually limited as there is no need for extra wet-lab procedures, there is just an extra analysis of the SNP raw data with an *in-house* developed algorithm.

With either technique, there are embryos that remain with no diagnosis or have an inconclusive diagnosis (Table 20). A statistically significant difference was found between the percentage of undiagnosed embryos analyzed with PCR and SNP_a ($p = 0.006$), highlighting that SNP_a has a higher diagnostic efficiency (around 97 %) than PCR (around 85 %), for the transport PGT cycles. This is in line with the latest ESHRE report (unpublished data), which details a successful genetic diagnosis for 95 % of analyzed embryos undergoing concurrent PGT-M/PGT-A testing whereas for PGT-M (which was PCR-based in 82 % of cases) the diagnostic rate was 88 %. Possible reasons for a no diagnosis or an inconclusive diagnosis result are contamination, ADO, recombination, and failure of amplification (Fig. 19). Even though no statistically significant differences were found in the percentage of embryos with no diagnosis due to ADO, recombination, or contamination between both test strategies, PCR-based diagnosis seems much more hampered by these events, compared to SNP_a. On the other hand, no diagnosis due to failure of amplification presented a statistical difference between PCR and SNP_a ($p = 0.012$).

Single-cell PCR tests are challenging and face numerous difficulties such as failure of amplification, contamination and ADO due to the low amount of DNA available [45, 59]. Contamination with extraneous DNA may lead to misdiagnosis. For instance, cellular DNA originating from sperm and/or maternal cumulus cells was considered a potential hazard. Therefore, the introduction of ICSI and removal of all cumulus cells before sperm injection represent suitable solutions to diminish the probability of contamination [45, 110]. Strict measures in the laboratory should be taken in order to diminish possible contaminations, since these problems can arise from the technicians or from carry-over of previous PCR products, this latter making the PCR strategy more prone to contamination than the SNP_a. In our transport PGT dataset, only two cycles presented embryos with no diagnosis due to contamination (both for PCR test strategy).

Amplification failure is another source of no diagnosis. In transport PGT, it was one of the major problems – it was not possible to diagnose 100 embryos due to amplification failure, which represents around 7 % of the total of embryos tested, and 72 % of the embryos without diagnosis. For this parameter, a clear difference is seen between both diagnosis strategies (Fig. 19). Single cells have a low amount of DNA, which might fail in amplifying due to intrinsic

factors, such as anucleate or degenerating cells. Lower quality embryos may also lead to a lower amplification efficiency [111]. Day 3 represents a crucial step in embryonic development as the embryonic genome is activated in this timeframe. Embryos which fail in genome activation may not be discriminated morphologically at day 3 but they may subsequently arrest in development and fail to reach the blastocyst stage [112]. This is reflected by the fact that the majority of embryos with no diagnosis after day 3 biopsy are not considered for rebiopsy at day 5/6 because of poor development and bad morphology. In comparison, amplification failure was a lesser problem for SNP_a. Several reasons may justify this result – first, the start amount of DNA is already higher, since 4 to 8 TE cells are biopsied; these cells undergo WGA through MDA, which generates products of more than 10 kb in length and yields sufficient DNA for several downstream tests [113]. Second, embryos which undergo TE biopsy have already been selected as the better-quality embryos.

ADO is one of the main obstacles to an accurate diagnosis, especially in the Multiplex-PCR approach and may lead to misdiagnosis. It is observed when only one of two alleles amplify, so it can only be detected in heterozygous samples. In transport PGT cycles, ADO was responsible for a ‘no diagnosis’ result in 31 embryos (around 2 % of the total of embryos tested with PCR), whereas SNP_a shows one isolated case of no diagnosis due to ADO. The number of genetic markers is usually more limited in Multiplex-PCR compared to SNP_a, making the latter strategy a more robust option. Despite the advantages of MDA, ADO also represents a setback with this technique [59, 114]. Suboptimal MDA reactions may yield higher ADO rates, and it might not be possible to give an accurate diagnosis.

5.2.2 Embryo transfer and pregnancies

In total, 200 transfers were performed, with 297 embryos, which resulted in 42 term pregnancies and 47 children, representing an overall LBDR of 21.0 % (Table 21). Most transfers were classified as ‘fresh’ transfers, but several factors must be considered on the ‘frozen’ transfers.

In most ART clinics, oocytes are fertilized after retrieval. Cryopreservation of oocytes is performed in special circumstances, such as lack of sperm on the day of fertilization [115]. Oocyte cryopreservation can also represent an option for couples with personal, religious or moral objections to embryo cryopreservation [116]. Historically, it is known that oocytes are difficult cells to cryopreserve, due to their low surface area to volume ratio and high susceptibility to form intracellular ice [117]. Advances in the cryopreservation methods, such as the use of vitrification rather than slow freezing strategies, resulted in better outcomes for oocyte cryopreservation. Preliminary safety data are reassuring, although large observational studies show that implantation rate and CPR are lower for frozen oocytes compared to fresh

or frozen embryos [118]. In transport PGT, 7 cycles of oocyte cryopreservation were done. In total, 28 oocytes were frozen, 13 survived the thawing process and were submitted to ICSI, five developed into embryos from which 3 were biopsied. One embryo was transferred but did not give a positive outcome.

Embryo cryopreservation at day 2 or 3 of development was performed in 60 cycles. No distinction between the day of cryopreservation was made since studies had shown the lack of differences in post-thawing survival rates between day 2 and day 3 frozen embryos [119]. Since most of these cycles resulted in a small number of embryos which lowered the chances of having an embryo suitable for transfer, cryopreservation was done at this stage with the purpose of accumulating embryos. These embryos were thawed in the next cycle and biopsied on day 3, often in combination with a cohort of fresh embryos. Post-thawed survival rate was not determined. This led in a number of cases to the transfer of a 'fresh' embryo that had been through a cryopreservation process ('FRET after D2/3 cryopreservation) or transferring a fresh embryo together with an embryo that had already been through a cryopreservation procedure, as a 'mixed transfer'. This type of transfers presented a higher CPR and LBDR (Table 21). Considering that, for the 'mixed transfer', it was not assessed which embryo implanted – the fresh or the one that had been cryopreserved – and that the number of transfers is very low for both categories, the conclusion about better outcomes may be incorrect. Nevertheless, we could speculate that the cryopreservation procedure prior to the biopsy does not seem to affect the pregnancy outcome. However, more data should be collected to strengthen this hypothesis. Cycles for embryo accumulation also created another scenario involving two cryopreservation procedures – one at day 2 or 3 of embryo development, prior to biopsy, and one after biopsy, at day 5. No pregnancies were achieved when an embryo cryopreserved twice was transferred, although positive outcomes have been reported in the literature [120].

Aizer *et al.* showed that the implantation rate and CPR are comparable between groups such as 'Fresh ET', 'FRET after PCR' 'FRET after D2/3 freeze' and 'FRET after D2/3 + D5 freeze', thereby concluding that the timing of embryo biopsy, either prior or post vitrification, has no negative impact on the potential implantation and pregnancy rates [121]. Our results seem to match with what was previously reported (Table 21).

To simplify the analysis, and since the numbers were very different between different types of transfers, only the 'traditional pathways' were assessed next – fresh transfer after a 2-blastomere biopsy followed by PCR, a frozen transfer after a 2-blastomere biopsy followed by PCR (embryos were cryopreserved at either day 5 or 6 of development) and a TE biopsy followed by SNP_a (Table 21). Implantation rate and CPR were much higher for SNP_a. In addition, a higher LBDR was observed for transfers after SNP_a. In 2013, strong evidence against blastomere biopsy approach arose after a publication from Scott Jr. and colleagues [122]. This team found out that a TE biopsy has no negative impact on the development of the

embryo competence (when measured as implantation and delivery rates). However, blastomere biopsy seems to significantly lower the probability of an embryo to implant and progress until the time of delivery. Therefore, TE biopsy appears to be a safer option when it comes to better clinical outcomes. This could be explained by the fact that it involves the removal of a smaller portion of the embryo's total cell content – a single blastomere removal on a cleavage-stage embryo represents around 13 % of the total cellular content; for transport PGT, 2 blastomeres were removed, which may represent up to 27 % of the total cellular content; in contrast, a 4 to 8 TE cell removal only represents 2 to 3 % of the total cellular content [123]. Another justifying reason is the fact the TE cells do not contribute to the fetus, whereas a blastomere is more likely to contribute to an embryonic cell lineage [124]. It is also plausible that the blastocysts have an increased tolerance to manipulation comparing with cleavage-stage embryos due to the fact that they have already undergone embryonic genomic activation [112]. Lastly, the *in vitro* development of embryos until day 5/6 allows a selection of better, competent embryos, as embryos that fail in genome activation will not further develop until blastocyst stage.

Additionally, it is important to note that TE biopsy followed by WGA and SNP_a allows for a comprehensive view of the whole genome. Therefore, it is possible to select embryos unaffected for the monogenic disorder, as well as euploid, which can also justify the better clinical outcomes observed. Several articles have suggested that the transfer of aneuploid and mosaic embryos can significantly decrease the success of the transfer. Mosaic embryos are characterized by the presence of two or more chromosomally distinct cell lines. It normally results from mitotic errors after fertilization, in the post-zygotic cell division [125, 126]. Several studies have shown that, although the transfer of mosaic embryos may result in a live birth, implantation rates are lower and miscarriage rates are increased in comparison with the transfer of euploid embryos [100, 125, 127-131].

5.2.3 Term and newborn characteristics

Within this dataset, 42 term pregnancies were achieved, 37 singleton and five twin pregnancies. It has been hypothesized that ART, especially invasive techniques as ICSI, can increase the risk of adverse maternal and neonatal outcomes. PGT adds the biopsy procedure, making it even more invasive than the IVF treatments alone [89]. A higher risk of preeclampsia and placenta previa has been reported, comparing IVF-no-PGT patients and IVF-PGT patients with a TE biopsy [88, 132]; also, a higher rate of cesarean section was described [133]. However, no differences between these two groups were detected in the gestational age of delivery, preterm birth, low birth weight, neonatal intensive unit admission, neonatal morbidities, or birth defects [88, 132]. Cleavage-stage biopsy seems to increase the risk of

gestational hypertension, when comparing with blastocyst biopsy, but no significant differences were described in perinatal deaths, birth defects, birth weight and gestational age [134]. Our results show that twin pregnancies have a higher incidence of preterm delivery and a lower birth weight compared to singleton pregnancies, as expected (Fig. 20 and 21). If we consider only singleton pregnancies, no statistically significant differences between the type of transfer and both the term of pregnancy and the birthweight were found (Fig. 22). Considering only the 'traditional' pathways, these comparisons also did not reach statistical significance (Fig. 23), thus confirming what has been previously reported by other authors.

5.3 Comparisons with *in-house* dataset

Transport PGT cycles between 2015 and 2019 were compared with *in-house* cycles, performed at UZ Brussels. Comparisons were made among 2-blastomere biopsy followed by Multiplex-PCR cycles and TE biopsy followed by SNP_a cycles.

Transport PGT cycles were previously characterized by a higher percentage of embryos not suitable for transfer due to aneuploidies detected with SNP_a rather than with PCR (Table 20). This difference was also observed when comparing the two techniques in the combined transport and *in-house* PGT cycles (Table 25).

Comparing transport and *in-house* PGT cycles, a statistically significant difference was found between the percentage of embryos with no diagnosis when tested with Multiplex-PCR ($p < 0.001$) but not when tested with SNP_a (Table 26). Empirically, it was known that transport PGT cycles had a higher incidence of no diagnosis due to ADO and failure of amplification, which is supported by evidence (Fig. 24). Proper shipping of the cryopreserved cells is a crucial step in transport PGT. Sample transport is done using dry ice (solid CO₂), which allows the maintenance of a constant temperature around $-70\text{ }^{\circ}\text{C}$. However, if ambient temperature raises, the dry ice can sublimate to gaseous CO₂ [135]. In case the PCR tubes were not correctly acclimatized, CO₂ may enter the tubes, creating a more acid environment, lowering the effectiveness of ALB, and consequently impacting subsequent DNA amplification. It has been suggested that problems in the lysis step can lead to higher rates of ADO [136]. As blastomeres are transported in ALB, if the PCR tubes are not correctly acclimatized, the lysis process may not be as efficient, causing higher rates of failure of amplification and ADO. This might be the reason why transport PGT cycles of blastomeres have higher rates of ADO and failure of amplification than *in-house* cycles ($p < 0.001$) (Fig. 24). On the other hand, this problem seems less important when transporting TE biopsied cells samples, since there is already a higher starting amount of DNA, and they are transported in PBS.

For pregnancy outcome comparisons, and to simplify the data analysis, only the 'classical' pathways of PGT were considered (Table 27). It is important to note that the number

of embryos transferred, and the number of transfers are very different between transport and *in-house* cycles. A total of 183 embryos diagnosed in transport PGT were transferred across 174 cycles, whereas 757 embryos diagnosed *in-house* were transferred across 715 transfers.

Lower implantation rate, CPR and LBDR were observed for the transport cycles. Several factors may be influencing these different rates – the quality/morphology of the embryos transferred (parameter that was not assessed), the age of the female, among others. It has been shown that the implantation rate of a transferred embryo, in IVF cycles, decreases linearly when the woman is over 35 years old [137]. A more recent study supports these findings, when transferring only euploid embryos. Also, it adds that, with increased age, a decreased implantation rate results in a lower CPR and LBDR, but the age of the female has no further impact on the progress of the implanted pregnancies to clinical ones and then to live birth, therefore suggesting that the main age-related obstacle to a successful pregnancy is implantation failure [138]. The age-related decrease of implantation rate remains unclear, but several reasons have been suggested as possible explanations, such as the uterine environment. Due to the advanced age, the uterus might not be able to supply all the requirements for the development of a pregnancy. An aging uterus becomes more fibrotic, there is a lower blood supply and the endometrium thickness is reduced, as well as its capacity to produce prostaglandins [139]. In transport PGT cycles, the median age of female at the moment of oocyte retrieval was 34 years old, meaning that lower implantation rates, and subsequent CPR and LBDR could be expected comparing with *in-house* cycles.

A day 5/6 biopsy followed by SNP_a presents higher rates than the day 3 biopsy followed by PCR, thus solidifying the results previously discussed on the transport cycles – TE biopsy seems to be a safer option, with less impact on further embryonic development [122] and, together with whole-genome diagnosis, SNP_a seems to yield better clinical outcomes. Transfer after a day 5/6 biopsy followed by SNP_a presents an average implantation rate of 58.84 % and a CPR per embryo transfer of 45.25 %, numbers in line with the latest ESHRE report (unpublished data).

Transport and *in-house* PCR cycles present a combined CPR of 29.0 %, which is lower but comparable to the 35 % CPR reported by ESHRE (unpublished data). Still, a noticeable difference was found between the implantation rate and CPR between fresh or frozen transfer after a blastomere biopsy followed by Multiplex-PCR. It is important to also make the remark that all blastocyst biopsies require a cryopreservation procedure before transfer, thus all embryos transferred were cryopreserved at day 5/6 of development. A recent study has shown that, in IVF patients, a higher CPR and LBDR was found for a frozen transfer at day 5/6 of embryo development [140, 141]. It has been suggested that a frozen transfer is favorable over a fresh transfer due to the adverse effects of controlled ovarian hyperstimulation (COH) in endometrial receptivity. 'Uterine receptivity' is defined as the state when the endometrium is

available for an embryo to implant. Three pre-requirements are essential for a successful embryo implantation – an embryo with implantation competence, a receptive endometrium and a synchronized development between the embryo and the endometrium. Uterine receptivity is better achieved during natural cycles; therefore, cryopreservation of embryos followed by transfer in a deferred cycle may be favorable to circumvent the damaging effects of COH in the embryo-endometrium synchrony [141, 142]. Besides that, data have shown other advantages of a frozen blastocyst transfer in IVF treatments, such as the lower risk of ectopic pregnancy, which is the main cause of maternal death in early pregnancy [143].

In-house cycles did not collect information on the birthweight of the children, so only the term of the pregnancy was assessed. Between the three types of transfer, no statistically significant differences were found for this parameter (Fig. 25). Therefore, there seems to be no increased risk of preterm birth after PGT treatment.

Lastly, prenatal test should be recommended to all PGT patients [94]. ESHRE Consortium strongly advises confirmatory prenatal testing to verify PGT results, since there is a risk for misdiagnosis due to technical limitations [144]. However, a very small percentage of PGT patients accept prenatal test. One of the strongest reasons to decline prenatal test is the patients concern on the risks of invasive tests, such as amniocentesis or CVS. Though, it has been reported that the risks of miscarriage after either amniocentesis or CVS are lower than those mentioned to women [145]. Also, the emotional, as well as the financial investment for PGT-M might also play a role in the unwillingness to undergo confirmatory diagnostic testing.

6. Final remarks

PGT has evolved over time, both in embryo biopsy techniques and diagnosis technology. A change from day 3 to day 5/6 biopsy has been observed, especially for concomitant analysis of PGT-M and PGT-A, while for PGT-M solely, this change has been happening slower. The implementation of genome-wide approaches avoids the need for locus-specific preclinical workups (usually done with Multiplex-PCR), thus reducing the workload but increasing the costs due to expensive equipment and consumables, making techniques such as SNP_a very hard to implement for many centers (unpublished data from ESHRE). Our data clearly shows numerous advantages of TE biopsy followed by genome-wide technology, in several timepoints of the procedure - preclinical workups tend to be ready faster, hence reducing the waiting time for the couple; SNP_a allows for concurrent analysis of PGT-M and PGT-A, thus allowing the transfer of non-affected and euploid embryos; SNP_a has a higher diagnostic efficiency, meaning that less embryos remain with no diagnosis, increasing the chances for a suitable embryo for transfer and lessening ethical and moral concerns. However,

it is important to acknowledge that whole-genome technology yields additional genetic findings, as chromosomal mosaicism, which raises other dilemmas on genetic counselling and embryo transfer policy. After transfer, higher implantation rate, CPR and LBDR are observed. The required frozen embryo transfer also seems to present benefits over fresh transfers. Finally, there seems to be no increased risk in preterm delivery and low birth weight. Therefore, a day 5/6 TE biopsy followed by a genome-wide technology for diagnosis, such as SNP_a, seems to be a superior strategy considering PGT treatment.

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