PGD (Pre Genetic Diagnosis) /PGS (Pre Genetic Screening)

Preimplantation genetic diagnosis (PGD) offers the possibility of a genetic diagnosis during an IVF cycle in order to increase the potential of a successful embryo transfer and implantation. It is a genetic test on the embryo cells that helps the embryologists select the best embryo(s) that are free of a genetic disease. Preimplantation genetic diagnosis (PGD) was developed due to the need to provide an alternative to prenatal diagnosis for couples that face the risk of transmitting a genetic disease to their children. Preimplantation Genetic Diagnosis and Screening (PGD/PGS) for monogenic diseases and/or numerical/structural chromosomal abnormalities is a tool for embryo testing aiming at identifying non affected and/or euploid embryos in a cohort produced during an IVF cycle. It is recognized as an important alternative to pre-natal diagnosis. Re-implantation embryo diagnosis requires in vitro fertilization, embryo biopsy with either using fluorescent in situ hybridization or polymerase chain reaction at the single cell level. In the late 1980s, many teams worldwide were attempting clinical PGD, including the Hammersmith team in London. In synthesis, PGD/PGS is a powerful tool to reach the goal of a pregnancy and attenuate its adverse events. In order to achieve this goal, it is mandatory not to significantly harm the embryo during the biopsy and to preserve its viability and reproductive potential.

Who should consider PGD?

PGD could be considered as an option in cases of:

- Couples with an increased risk for chromosome abnormalities or specific genetic diseases. This includes women who have had several miscarriages, or who have had a prior pregnancy with a chromosome abnormality.
- Women over the age of 38 and men with some types of sperm abnormalities may also produce embryos with higher rates of chromosome abnormalities. In addition, if a person carries a rearrangement of the chromosomes, PGD can identify which embryos have a normal amount of chromosomal material. When there is a 25% or 50% chance to have a child affected with a specific genetic disease, PGD can be designed to identify which embryos are affected, unaffected, or a carrier (if applicable) for that disease. Then, only

embryos without the disease are transferred to the uterus to attempt pregnancy.

Assisted reproduction and fetal genetic material in PGD

In the majority of the reported cycles, intracytoplasmic sperm injection (ICSI) is used instead of IVF. The main reasons are to prevent contamination with residual sperm adhered to the zona pellucida and to avoid unexpected fertilization failure. The ICSI procedure is carried out on mature metaphase-II oocytes and fertilization is assessed 16–18 hours after. The embryo development is further evaluated every day prior to biopsy and until transfer to the woman's uterus. During the cleavage stage, embryo evaluation is performed daily on the basis of the number, size, cell-shape and fragmentation rate of the blastomeres. On day 4, embryos were scored in function of their degree of compaction and blastocysts were evaluated according to the quality of the throphectoderm and inner cell mass, and their degree of expansion.

Cleavage Stage Biopsy

Cleavage stage biopsy is normally performed on day 3 embryos with at least 6 blastomeres. The zona pellucida is opened, and biopsy is mainly conducted following 3 methods of zona breaching, namely, laser-assisted, mechanical, and Tyrode's drilling. The Use of laser-assisted method represents 75% of all biopsy procedures. The main advantage of cleavage-stage biopsy over PB analysis is that the genetic input of both parents can be studied and leaves enough time to finish the diagnosis before the embryos must be replaced in the patient's uterus, it allows for the diagnosis of the embryos before day 5.

Polar Body Biopsy

Polar body (PB) biopsy on MII oocytes and/or zygotes was encouraged as a valuable alternative to blastomere biopsy. In some countries this was mainly due to legal reasons, since embryo biopsy is not allowed. PB biopsy is potentially less invasive than any other stage of preimplantation development, since it entails the removal of waste products of meiosis .however, the applicability of this strategy has always

been under debate, as mirrored by the ESHRE PGD Consortium data. In fact, PB biopsy has been used in only 10–15% of all the procedures performed in Europe in the last decade. Nowadays this rate is further decreasing probably due to the number of studies that highlighted technical, economical, biological, and clinical deficiencies underlying the approach. Mitotic and paternally derived aneuploidies cannot in fact be detected. No sufficiently powered well-controlled studies have been published that report a lack of PB biopsy impact upon embryo implantation potential. In light of this absence, the safety of the procedure still remains an arguable assumption. Guidelines regarding the proper timing for biopsy have not been established, it should occur between 8 and 14 hours after fertilization.

Morula Stage Biopsy

Recently morula stage biopsy has been proposed. Few data have been produced to evaluate its actual feasibility; however it is technically similar to cleavage stage biopsy.

Blastocyst Stage Biopsy

Blastocyst stage biopsy strategy was an important breakthrough in modern IVF. Several preclinical and clinical studies soon recognized its value, so that at present it is gradually replacing both cleavage stage and PB biopsy approaches. The power of TE (trophectoderm) biopsy resides in its higher technical and biological robustness. This approach in fact entails both lower influence of procedural errors and lower impact of mosaicism on the molecular analysis. Nevertheless, once a proper culture system is set, blastocyst culture itself elicits higher live birth rate per embryo transfer than cleavage stage .it is important to underline that also cleavage stage biopsy subtended culture to the blastocyst stage if aiming at performing a fresh embryo transfer of euploid embryos.

Aneuploidies

Studies strongly suggested that the age-related aneuploidies are mainly due to nondisjunction occurring during maternal meiosis. However, the study of first and second polar bodies with the help of one of the cytogenic methods, enable us to detect aneuploidy oocytes in IVF patients. Thus, it can help us to formulate a way to

prevent the transfer of embryos resulting from aneuploidy oocytes. It may reduce the chances of an IVF couple to have a child with Down syndrome and other common aneuploidies. With the help of FISH, the detection of chromosome signals in interphase nuclei is possible. Hence, this may be a reliable method for detection of common aneuploidies before the implantation takes place.

Genetic analysis techniques

The study of the genetics of cells and molecular biology has helped to build up some 'in situ hybridization' techniques, through which congenital disorders or abnormalities can be detected to handle the cases more effectively and efficiently during the clinical practices. The level of accuracy and specificity in these diagnoses play the pivotal role in treating, curing, preventing, and lessening the pain and agony of the patients as well as pave the way for further clinical developments in these fields of medical sciences. Fluorescent in situ hybridization (FISH) and Polymerase chain reaction (PCR) are the two commonly used, first-generation technologies in PGD. PCR is generally used to diagnose monogenic disorders and FISH is used for the detection of chromosomal abnormalities (for instance, aneuploidy).The detection of specific gene sequences on the chromosome, or either its presence or absence, is the central concern of cytogenetic technique in diagnosing as well as enumerating a genetic disorder or abnormalities. Among the tools of cytogenetic techniques, a technique named fluorescence in situ hybridization (FISH) was developed in the early 1980's. Applications of the FISH assay have been on the rise since the 1990's. Fluorescent deoxyribonucleic acid (DNA) probes, which are attached to the high degree of complementary parts of the chromosome, emit the colored signals. These signals could be grasped and visualized using fluorescent DNA probes, which further unveiled another way of detecting genetic abnormalities in medical science screening or chromosomal translocations When FISH is used to evaluate the genetic make-up of an embryo, the embryos are grown to the Day 3 stage, and a single cell is removed from each embryo. The cells are then attached to a glass slide, packaged and sent to the fertility clinic's genetic testing laboratory partner for evaluation. The FISH technique involves testing fragments of DNA that are specific to each chromosome. Probes (small pieces of DNA that are a match for the chromosomes being analyzed) are placed on the slide with the cell from the embryo and will attach to the chromosome target.

FISH, however, cannot fully access all the chromosomes — a human cell contains 23 pairs of chromosomes, but FISH analysis allows accurate assessment of only 10 to 12

chromosomes in each biopsied cell. This means that many abnormal embryos, incapable of forming a successful pregnancy, remain undetected and may be transferred. Chromosomes that can be analyzed with PGS (Pre Genetic Screening) include X, Y, 1, 13, 16, 18, and 21.

Polymerase Chain Reaction (PCR)

PCR, sometimes called DNA amplification, is used for the diagnosis of single gene defects, including dominant and recessive disorders. It is a technique in which a particular DNA sequence is copied many times in order to facilitate its analysis. PCR rapidly multiplies a single DNA molecule into billions of molecules. PCR requires sufficient amounts of a pure, high-quality sample of DNA, which is sometimes difficult to obtain from a single cell. The success of PCR in amplifying small quantities of DNA to a level at which they can be visualized and subjected to further genetic analysis has made the technique one of the most important diagnostic techniques in the modern molecular laboratory. Application of PCR protocols to single cell analyses has proved to be challenging but ultimately highly successful, and remains the only means of detecting specific mutant alleles in human preimplantation embryos.

Amplification efficiencies at the single cell level are generally lower than those encountered during the routine PCR of DNA samples in which the amount of starting template may be larger by several orders of magnitude. Reduced amplification efficiency can be the result of many problems encountered between sample collection and the PCR procedure itself. Operator problems such as cell loss during the delicate process of cell transfer to the tube or spontaneous cell lysis before the cell entering the tube contribute to amplification failure or reduced amplification efficiency. Intrinsic factors such as enucleate or degenerating cells with concomitant absence or degradation of DNA respectively are more difficult to control.

Allele Dropout: Another problem unique to single cell PCR is that of allele dropout (ADO), a phenomenon whereby only one of the two alleles present is successfully amplified. ADO is only detectable when heterozygous alleles are present but appears to be indiscriminate, in that the allele successfully amplified is random (even when only differing by a single nucleotide). ADO remains the biggest obstacle to accurate and efficient PGD for single gene disorders and the severity of its consequences is closely linked to the mode of inheritance of the disorder under test. For autosomal recessive conditions when both partners are carrying the same mutation, ADO should not, in the absence of contamination, result in the transfer of an affected

embryo. However, the number of embryos available for transfer will decrease as the ADO rate increases, potentially reducing the likelihood of pregnancy. In such cases, there is some reassurance in the calculation that a 10% allele dropout rate would only result in the exclusion of, on average, 2.5% of embryos for which a diagnosis was successfully made (based on a 90% amplification rate).

Real-time PCR: allows the rate of amplicon accumulation to be measured by detection of fluorescently tagged probes at each cycle of the reaction. The use of probes directed to either wild-type or mutant sequence also allows genotyping to be performed. The technique is rapid and has the added convenience that the amplification and detection procedures are carried out in the same tube (ie, as a homogeneous assay), thereby greatly reducing the chances of laboratory contamination

Single Nucleotide Polymorphism (SNP) Analysis

SNP is another newer technique that can examine all 23 chromosome pairs. SNPs are single bases, the building blocks of DNA, which can be in a different sequence in different individuals. For SNP microarrays, the technical strategy is a little different than for CGH.

Comparative Genomic Hybridization (CGH)

With CGH, the embryo nucleus is labeled with a fluorescent dye and a control cell is labeled using another color (ex. red or green). The ratio between the two colors is compared. If the chromosomal analysis shows an excess of red, the embryo nucleus contains an extra chromosome. If an excess of green is apparent, then the embryo nucleus is missing one of these chromosomes. CGH allows genetic specialists to examine all 23 chromosomes and provides a more detailed picture of the entire length of the chromosome, which may detect imbalance of chromosomal segments. The CGH technique takes approximately 72 hours, which means embryos must be frozen to provide the time necessary to obtain a diagnosis. A newer advanced technique offered in some labs is called array CGH or microarray CGH (mCGH). This is an accelerated CGH protocol providing results in 24 hours for all chromosomes.

Clinical practice

The experience of Medical Genetics Laboratory has been practiced in PGD for monogenic diseases over the last 13 years, in the Medical laboratory of the National and Kapodistrian University of Athens, such as:

- Mediterranean hemopathic syndromes
- Cystic fibrosis(CF)
- Superior mesenteric artery syndrome(SMA)
- X-linked recessive inheritance as Duchenne muscular dystrophy
- The fragile X syndrome
- (Congenital Lipoid Adrenal Hyperplasia – CLAH).

Also we examined and dealt with rare diseases such as:

- Glycogen Storage Disease – GSD
- Spastic paraplegia 3A (SPG3A)
- Leber's Congenital Amaurosis - LCA
- Marfan syndrome (MFS)
- Autosomal recessive polycystic kidney disease (ARPKD)
- Neurofibromatosis type I (NF-1)
- Myotonin-protein kinase(DM1)

All the above diseases are having conspicuous signs and symptoms, but instead all that CF relates with males infertility. The Greek population of men, reaches the 70% of congenital absence of vans deference and the remaining 30% suffers by obstructive azoospermia. Mediterranean syndromes and CF are two of the most common diseases in the Greek population. 10% of the population are carriers of β-thalassemia, (Mediterranean anemia or Cooley anemia), hemoglobinopathies, such as sickle cell anemia or drepanocytosis and 5% are carriers of CF. More than 80 mutation have been detected into CFTR gene , which is responsible for CF and hemoglobin beta gene. Based on the data the 85% of the CFTR gene mutations are located in 7 of the 27 gene exons and 97% in the hemoglobin beta gene are located in the first half of the gene, we designed and improved two flexible and easily adopted protocols.

Mediterranean syndromes

For Mediterranean syndromes, the methodology we apply is based on the PCR amplification of the first half of the hemoglobin beta gene, in which are identified most of the mutant mediated mutations. At the same time three polymorphic microsatellite sequences are propagated in association with beta gene. The detection of mutations are performed with nested PCR in real-time PCR (system of LightcyclerTM Roche) while polymorphic sequences are analyzed by fluorescent dye-labeled primers fragmentation (fragment analysis). By estimating the sizes between parent and fetal alleles (linkage analysis) an indirect diagnosis is made while at the same time the probability of contaminations of aberrant DNA in each individual cell is controlled. The method ensures the diagnosis of 95% of the mutations in Greek population within 6 to 7 hours for biopsy of the collected cells, and this can be applied to other populations too.

Cystic fibrosis

For this genetic disorder the protocol that is designed permits according to candidate's genotype, the proliferation in a PCR (multiplex PCR) of any combination of the 7 exons of the CFTR gene (4, 10, 11, 13, 14b17b, 21) together with two polymorphic intragenic microsatellites sequences in introns 1 and 8. Then exons are separately multiplied by nested PCR and analyzed by electrophoresis on acrylamide gel with increasing concentration of denaturing agents (DGGE). Microsatellite sequences are analyzed fluorometrically by electrophoretic separation. This protocol assures the genetic diagnosis of more than 75% combinations of genotypes existing in Greek population. Since the microsatellite sequences are factual, indirect diagnosis can be done and can be detected the possibility of foreign DNA infection.

Other diseases

The PGD for chromosomal aberrations, was designed a uniqueness multiplex PCR, in which 9 different genetic types of genosomes have been multiplied. The method allows the determination of sex in a cell. At the same time, depending on the disease of interest each time, the multiplication is incorporated and the part of the gene associated with the particular disease, such as the dystrophin gene or the STAR gene (Duchenne muscular dystrophy or lipid adrenal hyperplasia, respectively).the PGD protocol for spinal muscular atrophy (SMA) is based on proliferation of the normal

SMN protein by allele specific PCR, known as ARMS. At the same time two microsatellite polymorphic sequences, from right and left side of gene are multiplied, so indirect confirmation can be done for the existence of the normal gene and contamination control at the same time.

In any case of other monogenic diseases (glycogenosis, Leber congenital malaria, spastic paraplegia, Marfan syndrome, autosomal recessive polycystic kidney disease and type 1 neurofibromatosis) ,Based on the mutations of the candidate parents who desired PGD, part of the gene multiplies in which the parental exchanges are located and linked with at least two polymorphic microsatellite sequences. In case of myotonic dystrophy type 1, corresponding to the protocol applied concerns the proliferation of the normal allele from each parent, as the pathological allele is too large to be detected by simple PCR. The use of two microsatellite sequences in conjunction with the DMPK gene also allows for indirect confirmation of the diagnosis, as in previous protocols. During the last 13 years it is observed annually in the laboratory of Medical genetics ,the number of couples who preferred the IVF process in conjunction with the PGD have increased getting over the 60 cycles per year. During that period, 371 couples were asked to be informed about PGD process and at least 287 of them have started one cycle,a total of 494 cycles.

In the 426/494 cycles, PGD was finally performed because over 25-30% of the cycles were interrupted due to the reduced of hormonal response, with few or poor quality ova. Genetic diagnosis was performed either in blastocoel or by cells from the outer layer of blastocyst. Genetic diagnosis was achievable in more than 93% of the biopsied embryos. Until now 156 healthy newborns have been born.

Ethical, Legal and Social Issues Relating to PGD

Considerable differences in the regulatory oversight of PGD exist among countries, ranging from total bans on any embryo manipulation to the almost complete absence of any regulations or authority. The high cost of practice, low pregnancy rate, problems with patient access and insurance coverage appear to be the biggest drawbacks to universal acceptance in societal terms. Ethical discussions considering the moral status of the human embryo and what constitutes severe genetic disease have been debated elsewhere but such discussions are clearly outside of the purview of this methodological review. Somewhat reassuring for those centers currently

offering PGD is the acknowledgment from professional organizations that PGD can now be considered a "standard of care" rather than an experimental treatment

Who can benefit from PGD?

Preimplantation genetic diagnosis can benefit any couple at risk for passing on a genetic disease or condition.

The following is a list of the type of individuals who are possible candidates for PGD/PGS:

- Carriers of sex-linked genetic disorders
- Carriers of single gene disorders
- Those with chromosomal disorders
- Women aged 35 and over
- Women experiencing recurrent miscarriages
- Women with more than one failed fertility treatment

PGD has also been used for the purpose of gender selection. However, discarding embryos based only on gender considerations is an ethical concern for many people.

The following are considered benefits of PGD/PGS:

- PGD can test for more than 100 different genetic conditions.
- The procedure is performed before implantation thus allowing the couple to decide if they wish to continue with the pregnancy.
- The procedure enables couples to pursue biological children who might not have done so otherwise.

The following are considered concerns or disadvantages associated with the use of PGD/PGS:

- Many people believe that because life begins at conception, the destruction of an embryo is the destruction of a person.
- While PGD helps reduce the chances of conceiving a child with a genetic disorder, it cannot completely eliminate this risk. In some cases, further testing is needed during pregnancy to ascertain if a genetic factor is still possible.
- Although genetically present, some diseases only generate symptoms when carriers reach middle age. The probability of disorder development should be a topic of discussion with the healthcare provider.
- Keep in mind that preimplantation genetic diagnosis does not replace the recommendation for prenatal testing.