Preimplantation Genetic Testing for monogenic disorders
Martine De Rycke
Outline

- overview of PGT
- embryo biopsy
- PGT for monogenic disorders
Preimplantation Genetic Testing

- A test performed to analyse the DNA from oocytes (polar bodies) or embryos (cleavage stage or blastocyst) for HLA-typing or for determining genetic abnormalities

- PGT for aneuploidies (PGT-A)
- PGT for monogenic/single gene defects (PGT-M)
- PGT for chromosomal structural rearrangements (PGT-SR)

International glossary on fertility and infertility care
Zegers-Hochschild et al., Human Reproduction, 2017
PGT-A

- Aneuploidy testing involves selection of euploid embryos

- For couples at high genetic risk
  (example: XXY or mosaic 45,X/46,XX)

- To improve IVF results for specific IVF patients groups at low genetic risk
  (advanced maternal age, recurrent IVF failure or repeated miscarriages)
PGT-A for IVF patients

- v 1.0: no benefit (improvement of live birth rate) with FISH/ blastomere from cleavage stage biopsy (meta-analysis of RCTs, Mastenbroek et al., 2011)

  technical: possible negative impact of cleavage-stage biopsy and limitations of FISH

  biological: high chromosomal mosaicism in the early embryo
PGT-A for IVF patients

- v 1.0: no benefit with FISH/cleavage stage biopsy (meta-analysis of RCTs, Mastenbroek et al., 2011)

- v 2.0: arrayCGH/NGS and blastocyst biopsy study of Rubio et al., 2017
  AMA group, arrayCGH, cleavage stage,
  => fewer ET
  lower miscarriage rates
  reduced TTP
  better delivery rate at first attempt
  no benefit for cumulative delivery rate
PGT-M and PGT-SR

- for couples at *high* risk of transmitting a genetic condition to their children (often fertile couples)

- **PGT-SR:**
  - Robertsonian translocations
  - Reciprocal translocations

- **PGT-M:** any single gene disorder for which causative mutation has been identified
  - Autosomal recessive, dominant or X-linked
PGT and HLA typing

- Human Leucocyte Antigens (HLA) typing
  aim: establish a pregnancy which is HLA compatible with a sick sibling, so that at birth, cord blood can be collected and used for haematopoietic stem cell transplantation and cure of the sick child

→ HLA-typing alone for acquired disease
  25% of biopsied embryos are genetically transferable

→ HLA-typing in combination with mutations underlying immunodeficiencies or hemoglobinopathies
  19% of biopsied embryos are genetically transferable for an AR disorder (1/4 x3/4)


**Sexing of Live Rabbit Blastocysts**

R. G. EDWARDS & R. L. GARDNER

Physiological Laboratory, University of Cambridge
History of PGT

Human IVF in 1978
R. Edwards: Nobel prize for medicine in 2010

PCR in 1983
K. Mullis: Nobel prize for chemistry in 1993
History of PGT

- 1990: Handyside et al.: first PGD for X-linked disease
- 1992: Handyside et al.: baby after PGD for Cystic Fibrosis

Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification

A. H. Handyside, E. H. Kontogianni, K. Hardy & R. M. L. Winston
Institute of Obstetrics and Gynaecology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 ONN, UK

OVER 200 recessive X chromosome-linked diseases, typically affecting only hemizygous males, have been identified. In many of these, prenatal diagnosis is possible by chorion villus sampling (CVS) or amniocentesis, followed by cytogenetic, biochemical or molecular analysis of the cells recovered from the conceptus. In others, the only alternative is to determine the sex of the fetus. If the fetus is affected by the defect or is male, abortion can be offered. Diagnosis of genetic defects in preimplantation embryos would allow those unaffected to be identified and transferred to the uterus. Here we report the first established pregnancies using this procedure, in two couples known to be at risk of transmitting adrenoleukodystrophy and X-linked mental retardation. Two female embryos were transferred after in vitro fertilization (IVF), biopsy of a single cell at the six- to eight-cell stage, and sexing by DNA amplification of a Y chromosome-specific repeat sequence. Both women are confirmed as carrying normal female twins.
History of PGT

• 2001: Verlinsky et al.: first ‘saviour baby’
Belgian law

Art. 67. Verboden zijn:
1° Genetische pre-implantatiediagnostiek met het oog op eugene
cine selectie, zoals gedefinieerd in artikel 5, 4°, van de wet van 11 mei 2003
betrokken het onderzoek op embryo's in vitro, dat wil zeggen gericht op de
selectie of de verbetering van niet-pathologische genetische
kenmerken van de menselijke soort;
2° Genetische pre-implantatiediagnostiek met het oog op
geslachtsselectie, zoals gedefinieerd in artikel 5, 5°, van de wet van 11
mei 2003 betrokkens het onderzoek op embryo's in vitro, dat wil zeggen
gericht op geslachtsselectie, met uitzondering van de selectie ter
voorkoming van geslachtsgebonden ziekten.

Art. 68. In afwijking van artikel 67 is pre-implantatie genetische
diagnostiek uitzonderlijk toegestaan in het therapeutisch belang van een
reeds geboren kind van de wensouder(s).
Het geraadpleegde fertiliteitscentrum moet, in het geval bedoeld in het
eerste lid van dit artikel, beoordelen of de kinderwens niet uitsluitend ten
dienste staat van dat therapeutisch belang.
PGT and HLA matching
PGT involves a multidisciplinary team

Close collaboration between IVF and diagnostic genetics unit:

10/40 in-house cycles
16/40 transport cycles
14/40 in-house and transport cycles

(ESHRE PGT Consortium survey 2013-2015, unpublished)
PGT workflow in daily practice

- Intake and evaluation of PGT request
  - is PGT acceptable, is PGT possible?
- Consultation at the IVF/genetics unit
  - counselling and informed consent, sample collection
- Pre-PGT workup in the genetics lab
- PGT clinical cycle
- Follow-up
  - of cycles, pregnancies and children
PGT workflow in daily practice

- intake and evaluation of PGT request
  - is PGT acceptable, is PGT possible?
- consultation IVF and genetics unit
  - counselling and informed consent,
- pre-PGT workup in the genetics lab
- PGT clinical cycle
- follow-up
PGT clinical cycle

- Oocyte collection after hormonal stimulation (day 0)
- IVF with Intracytoplasmic sperm injection (day 0)
PGT clinical cycle

**in vitro culture**

- **cleavage stage (day 3)**
- **morula stage (day 4)**
- **blastocyst stage (day 5/6)**

[Images of cell stages and blastocoele diagram]
Embryo biopsy is a two step process:

1) punction or removal of part of the ZP
   - mechanical opening (needle)
   - chemical opening (Acidic Tyrode)
   - laser pulses (most common)

2) removal of nucleated cell(s)
PGD clinical cycle: biopsy

1 or 2 polar bodies from oocytes
day 0/1

1 or 2 blastomeres from cleavage stage embryos
day 3

5-10 trophectoderm cells from blastocysts
day 5/6
PGT clinical cycle: day 3 biopsy

blastomere(s) from cleavage stage embryos (day 3):

- for maternally and paternally inherited conditions
- gender determination possible
- inherent high chromosomal mosaicism rate
- impact of 1 or 2 embryonic cell removal on embryonic development/implantation?
PGT clinical cycle: day 3 biopsy

RCT: 8-cell embryos on day 3
single embryo transfer on day 5
De Vos et al., 2009

live birth delivery rates: 37.4 % (1 cell)
22.4 % (2 cells)
35.0 % (control)

clinical outcome of 1-cell biopsy was significantly better than that of 2-cell biopsy
PGT clinical cycle: ‘fresh embryo transfer’

- **COS biopsy day 3**
- **genetic testing results day 5**
- **(single) fresh transfer day 5**
- **cryo day 5/6**

**surplus genetically transferable embryos**
### PGT clinical cycle: ‘freeze all strategy’

<table>
<thead>
<tr>
<th>COS</th>
<th>biopsy day 3</th>
<th>genetic testing results day 5</th>
<th>fresh transfer day 5</th>
<th>cryo day 5/6</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>all genetically transferable embryos transfer in later non-stimulated cycle</td>
</tr>
</tbody>
</table>

- **risk OHSS**
- **endometrium receptivity**
PGT clinical cycle: ‘freeze all strategy’

- biopsy day 3
- genetic testing results day 5
- fresh transfer day 5
- cryo day 5/6

all genetically transferable embryos transfer in later non-stimulated cycle

better synchronicity between embryo and endometrium
PGT clinical cycle: TE biopsy

TE cells from blastocyst embryos (day 5/6):

- extraembryonic cells / safer option
- no negative impact on implantation
- multiple cells / higher diagnostic accuracy
- lower chromosomal mosaicism level
- less samples for testing
- need for good blastocyst culture systems
- need for vitrification and transfer in natural cycle
PGD clinical cycle: TE biopsy

- Double embryo transfer
- Non-biopsied
- d3 or d5 biopsy
- SNP array profiling
- Singleton babies: identify which embryo implanted
- d3: 11/13 non-biopsied
- d5: 9/16 non-biopsied

Scott et al., 2013
PGT clinical cycle: TE biopsy

TE cells from blastocyst embryos (day 5/6):

- extraembryonic cells / safer option
- no negative impact on implantation

- multiple cells / higher diagnostic accuracy
- lower chromosomal mosaicism level
- less samples for testing
- need for good blastocyst culture systems
- need for vitrification and transfer in later cycle
PTG clinical cycle: TE biopsy

- Biopsy (day 5/6)
- Cryo (day 5/6)
- Genetic testing results (day x)
- Keep genetically transferable (cryo) and discard non-transferable embryos
PGD clinical cycle: biopsy

Day 1: Polar Body Biopsy
Day 2: Cleavage stage biopsy
Day 3: Morula stage biopsy
Day 4: Blastocyst biopsy
Day 5: Trophoderm
Day 6: Blastocoelic fluid

Courtesy C. Magli
PGD clinical cycle: biopsy

OOCYTE / EMBRYO BIOPSY

Day 1  Day 2  Day 3  Day 4  Day 5  Day 6  Day 7

Polar Body Biopsy
Cleavage stage biopsy
Morula stage biopsy
Blastocyst biopsy
Trophectoderm
Blastocoelic fluid

intact 2-6 cells
fresh ET

Courtesy C. Magli
PGD clinical cycle: biopsy

-OOCYTE / EMBRYO BIOPSY

Day 1: Polar Body Biopsy
Day 2: Cleavage stage biopsy
Day 3: Morula stage biopsy
Day 4: Blastocyst biopsy
Day 5: Trophectoderm
Day 6: Blastocoelic fluid
Day 7

Intact 2-6 cells fresh ET
Minimally invasive origin of cell-free DNA

Courtesy C. Magli
PGD clinical cycle: biopsy

OOCYTE / EMBRYO BIOPSY

Day 1  Day 2  Day 3  Day 4  Day 5  Day 6  Day 7

Polar Body Biopsy

Cleavage stage biopsy

Morula stage biopsy

Blastocyst biopsy

Trophectoderm

Blastocoelic fluid

intact 2-6 cells fresh ET

minimally invasive origin of cell-free DNA

non-invasive spent embryo culture medium

Courtesy C. Magli
PGT clinical cycle: switch in biopsy/testing

cleavage stage biopsy (day 3) ↓

TE biopsy (day 5/6)

targeted testing (FISH, PCR) ↓

first step = whole genome amplification (WGA)

genome-wide testing various platforms (arrayCGH, low coverage NGS, SNParray)
PGT-M

targeted single cell PCR (STRs)

WGA

→ targeted regular PCR (STRs)

→ genome-wide SNP array (SNPs)

SNParray
PGT-M: PCR >> SNPArray

STR: Short Tandem Repeat
variation in number of 2, 3 or 4 bp
repeats -> many alleles
limited # of STRs in target region

SNP: Single Nucleotide Polymorphism
variation of 1bp,
SNP -> two alleles (A,B)
Karyomapping array with
300,000 SNPs genome-wide
PGT-M: PCR >> SNParray

Principle: alleles of mutation and genetic markers located near each other on the same chromosome will be inherited together.

Selection of informative markers during workup
PGT-M workup: informativity testing

STR genotyping to select STRs which are informative, allowing to distinguish alleles from each other.
PGT-M workup: informativity testing

(AD disorder)

<p>| | |</p>
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</table>

Informative:

Non-Informative
PGT-M workup: segregation analysis

Principle: alleles of mutation and genetic markers located near each other on the same chromosome will be inherited together.

Parental haplotypes are established during workup with family samples.
**PGT-M workup: segregation analysis**

Principle: alleles of mutation and genetic markers located near each other on the same chromosome will be inherited together.

<table>
<thead>
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<th>Father</th>
<th>Mother</th>
<th>Affected Child (AR)</th>
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<tr>
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Parental haplotypes are established during workup with family samples.
PGT-M: PCR >> SNParray

Principle: alleles of mutation and genetic markers located near each other on the same chromosome will be inherited together.

Known parental haplotypes are used for embryo diagnosis.

(AR disorder)
PGT-M workup: segregation analysis

(AD disorder)

Informative: A= mutant
Informative: B= mutant
Non-Informative
Non-Informative
Informative: B= mutant
PGT-M: PCR >> SNPArray

analysis of only flanking genetic markers
(known parental haplotypes from workup with family samples)

father

\[ \begin{array}{c|cc}
A & A & A \\
A & A & B \\
A & * & A \\
A & B & B \\
\end{array} \]

mother

\[ \begin{array}{c|cc}
A & A & B \\
A & B & A \\
B & A & A \\
B & B & A \\
\end{array} \]

embryo

\[ \begin{array}{c|cc}
A & B & A \\
A & A & A \\
B & A & * \\
B & B & B \\
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(AR disorder)
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</table>
PGT-M: PCR >> SNParray

- **indirect diagnosis**: use of genetic markers that flank the locus/gene of interest (within 1-2 Mb) more markers at either side make the test more robust
- when mutation analysis is not possible (region too large for amplification, pseudogene interference, …)
- **limiting factor**: informative couples with family history and requirement for samples form affected family members

no family samples/*de novo* mutation?
**PGT-M: PCR >> SNPArray**

**direct diagnosis:** add mutation detection to STRs/SNPs and establish haplotypes during PGD cycle(s), based on affected embryos (only for point mutations, small dups and dels)

- small dups and dels => differences in fragment length
- point mutations => post-PCR reactions for further allele discrimination
  → restriction enzyme analysis, ARMS, minisequencing….
PGT for sickle-cell anemia

c.20A>T in HBB

genomic DNA of carrier mother

genomic DNA of carrier father

genomic DNA of affected child

cell from a carrier embryo (E1)

cell from a carrier embryo (E3)

cell from homozygous normal embryo (E5)
PGT-M: single cell PCR vs WGA

- PGT-M haplotyping
  - targeted single cell PCR (STRs)
  - genome-wide SNP array (SNPs)

- WGA
Single/few cell targeted multiplex-PCR

- **Regular PCR**: 100-500 ng purified genomic DNA

- **Single-cell PCR**: lysed single cell with 6 pg or 2 DNA copies requires extensive optimisation of PCR conditions
  - **specificity**: primer design, hot start PCR, < 45 cycles
  - **contamination**: from carry-over or extraneous DNA
  - **allele drop out**: random amplification failure of one of two alleles in a single/few heterozygous cell(s) – may affect up to 5% of single-cell PCRs
Single/few cell targeted multiplex-PCR

- thermal cycling reaction with fluorescently labelled primers: fragment length detection on automated sequencers (fragments up to 400 bp)
- simultaneous amplification of multiple loci per cell (flanking genetic markers +/- mutation locus)

=> more accurate: allows diagnosis AND reveals contamination & ADO

capillary electrophoresis
5 fluorochromes
Single/few cell targeted multiplex-PCR
Whole genome amplification

from 6 pg (single cell) to several μg of DNA

amplification problems =>

bias
allele drop-out (ADO) and preferential amplification (PA)
amplification errors
incomplete genome coverage

different protocols (PCR-based or MDA-based)
⇒ different downstream applications

PGT-M: MDA-based WGA
PGT-A/PGT-SR: PCR-based WGA
Whole genome amplification: MDA

- Multiple Displacement Amplification, (MDA) isothermal amplification (30°C) => DNA fragments up to 70 kb, low error rates

Dean et al., 2002
Whole genome amplification: MDA

- Multiple Displacement Amplification, (MDA)
  - relative good genome coverage
    (except telomere and centromere repeats)
  - amplification bias
    (random under or overrepresentation of sequences)
  - chimeric amplification products (branching)
  - higher ADO and PA than in targeted PCR amplification (single cell)
PGT-M: SNParray

TE biopsy

SNP array
- generic, off the shelf
- standard workflow
- no workup of the test
- short workup of family
- double indications (HLA)

information on
- haplotypes
- chromosomal copy number
SNParray: PGT-M +?

- haplotypes + chromosomal copy number

- possible to detect some occurrences of aneuploidy with the Illumina karyomapping software, but not all → PGT-A is not a supported application

- extending SNP array with additional algorithms in-house algorithm, haplarithmisis from Zamani et al, 2015): combine haplotyping and chromosomal copy number
Overview

<table>
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<th>aCGH</th>
<th>low coverage NGS</th>
<th>karyomapping /SNPa</th>
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</table>
PGT team of UZ Brussel

Centre for Reproductive Medicine
- IVF team
- Biopsy team
- Fertility specialists  Prof. W. Verpoest
- Embryologists
- Prof. H. Tournaye

Centre for Medical Genetics
- DNA and cytogenetics lab
- Clinical geneticists
- PGD coordinator: P. De Becker
- Prof. M. Bonduelle

PGT lab team:
- Technicians
- V. Berckmoes
- P. Verdyck
- M. De Rycke
PGT-M: SNParray

1) identify informative SNPs in region of interest

2) haplotyping of SNPs in embryo vs reference
## SNP array: interpretation

2) phase SNPs in embryo vs reference

<table>
<thead>
<tr>
<th>father</th>
<th>mother</th>
<th>reference</th>
<th>embryo</th>
<th>phase</th>
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<tbody>
<tr>
<td>AB</td>
<td>AA</td>
<td>AB</td>
<td>AB</td>
<td>in phase</td>
</tr>
<tr>
<td>AB</td>
<td>BB</td>
<td>BB</td>
<td>AB</td>
<td>out of phase</td>
</tr>
<tr>
<td>AA</td>
<td>BB</td>
<td>not informative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB</td>
<td>AA</td>
<td>AA</td>
<td>AA</td>
<td>in phase</td>
</tr>
<tr>
<td>AB</td>
<td>AA</td>
<td>AB</td>
<td>AA</td>
<td>out of phase</td>
</tr>
<tr>
<td>AB</td>
<td>BB</td>
<td>AB</td>
<td>BB</td>
<td>out of phase</td>
</tr>
<tr>
<td>AB</td>
<td>AB</td>
<td>not informative</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

examples: informative allele in blue
SNP bead array preparation
SNP bead array: workflow

MDA based

1. Whole genome amplification and fragmentation
2. Denaturation and hybridisation on BeadChip
3. Single base extension and staining
4. Array scanning and genotype scoring
SNP array: principle

LaFramboise T, 2009
SNP array: principle

A = A/T base
B = G/C base
SNP bead array

A = A/T base
B = G/C base
NC = no call
PGT-M workup: segregation analysis

STR 1
- ♂: 7 repeats, p.F508del
- ♀: 9 repeats, wild type
- ♂: 10 repeats, wild type
- ♀: 14 repeats, wild type
- Affected child: 7 repeats, p.F508del

STR 2
- ♂: 11 repeats
- ♀: 11 repeats, 9 repeats
- ♀: 16 repeats
- Affected child: 11 repeats

Wild type
p.F508del
PGT: accuracy and safety

- general misdiagnosis rate < 1% (Wilton et al., 2009)
  possible causes: human & technical errors, intrinsic factors

- PGT children: follow-up results are reassuring
  d3 biopsy does not introduce extra risks (995 children, Desmyttere et al., 2012)
  neonatal outcome is similar between PGT (day 3 biopsy) and ICSI group (89 children, Hasson et al., 2017)

need for follow-up after d5 biopsy and long-term follow-up