Thursdays Webinars

EuroBloodNet

Preimplantation Genetic Testing for Rare Anaemias

Speaker Marina Kleanthous
Head of Molecular Genetics Thalassaemia Department
The Cyprus Institute of Neurology and Genetics
The Cyprus School of Molecular Medicine
ERN-EuroBloodNet subnetwork rare anaemias
Nicosia – Cyprus
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European Reference Network for rare or low prevalence complex diseases
Network Hematological Diseases (ERN EuroBloodNet)

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Conflicts of interest

I have nothing to declare.

Learning objectives of the webinar

1. To understand how PGT-M works

2. To learn how to develop a genetic test for PGT-M

3. To discuss the use of PGT-M and HLA typing for rare anaemias

Outline

- Introduction
- Preclinical work-up
- PGT stages and clinical cycle
- Cyprus results
- HLA typing and PGT-M for rare anaemias

PREIMPLANTATION GENETIC TESTING (PGT)

PGT is a reproductive option for couples at risk of having a child with an inherited genetic disorder.

DNA from oocytes or embryos biopsies is analysed in order to determine genetic abnormalities and identify unaffected embryos

PGT gives to the couple the chance to have an unaffected child without undergoing termination of pregnancy

- ✓ First PGT (*Handyside et al, 1990*)
- ✓ Thousands of babies have been born following PGT

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(http://www.eshre.eu/)

STAGES

- Patient inclusion/exclusion
- Counseling – Informed consent
- Preclinical work-up
- Induction of ovulation
- Oocyte collection
- Fertilization by ICSI
- Embryo culture and biopsy
- Genetic testing
- Implantation of one suitable embryo
- Confirmation of pregnancy
- Post-examination process

PGT

- Aneuploidies (**PGT-A**)
- Chromosomal structural rearrangements (**PGT-SR**)
- Monogenic disorders or single gene defects (**PGT-M**)
  - PGT-M can be offered for all monogenic disorders for which the disease-causing loci have been unequivocally identified
  - Nuclear (X-linked, autosomal, dominantly or recessively inherited)
  - Mitochondrial (maternally inherited) and involve (likely) pathogenic genetic variant(s)
- HLA typing with or without concurrent testing for a monogenic disorder

PGT-M Conditions

- **For high risk and serious disorders**
- PGT genetic testing can currently be used to avoid over 600 genetic conditions.
- For some of these conditions, pre-implantation HLA typing can be used
- National authorities are responsible for approving the conditions for PGT
- Human Fertilization and Embryology Authority (HFEA) in UK for PGT and HLA typing

HFEA: Human Fertilization and Embryology Authority in UK (https://www.hfea.gov.uk/)

Ethical and Legal Issues

The main concern in PGT is the fear of eugenics

- The provision of PGT is legally restricted in many countries[1]
- Policies and regulations vary from country to country
- In two out of 43 European countries PGT is not allowed (Malta and Bosnia & Herzegovina)[2]
- Many countries have a legislation banning any form of **eugenic selection**, allowing to select against **high risk and serious disorders**

1. Ginosa and Isaki, 2019. Regulating preimplantation genetic testing across the world: A comparison of international policy and ethical perspectives. Cold Spring Harb. Perspect. 2019, 10
2. Calhaz-Jorge et al., 2020. Survey on ART and IUI: Legislation, regulation, funding and registries in European countries: The European IVF-monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE) Hum. Reprod. Open 2020

PGT-M Stages

PGT Cycle

- Patient inclusion/exclusion
- Counseling – informed consent
- Preclinical work-up
- Induction of ovulation
- Oocyte collection
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Health professionals:
- Medical Geneticist
- Obstetrician
- Embryologist
- Molecular Geneticist

Preclinical Work-up

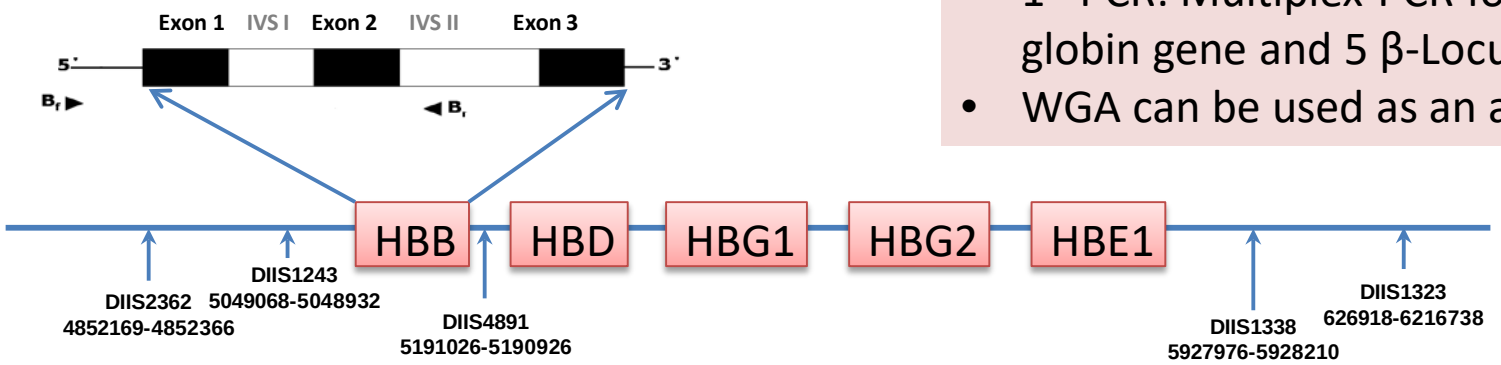
- The molecular genetic report of the disease is obtained
- Blood sample from appropriate family members are collected
- **Development of the 1st PCR amplification (multiplex targeted PCR or WGA)**
- **Development of the 2nd genetic test (targeted or generic)**
- Selection of testing strategy:
  - Targeted amplification of informative markers with or without the pathogenic variant(s)
  - WGA followed by targeted amplification of informative markers with or without the pathogenic variant(s)
  - WGA followed by a generic method such as SNP array or NGS (Bioinformatics support)
- Validation of the genetic testing

Whole genome amplification (WGA) by multiple displacement amplification (MDA) is recommended for PGT-M

Preclinical Work-up

Targeted amplification of informative markers with the pathogenic variant(s)

- Selection of at least 4 STR markers flanking the gene of interest (publications or databases)
- Primer design for the flanking STR markers and the region containing the pathogenic variant
- Development of PCR assays



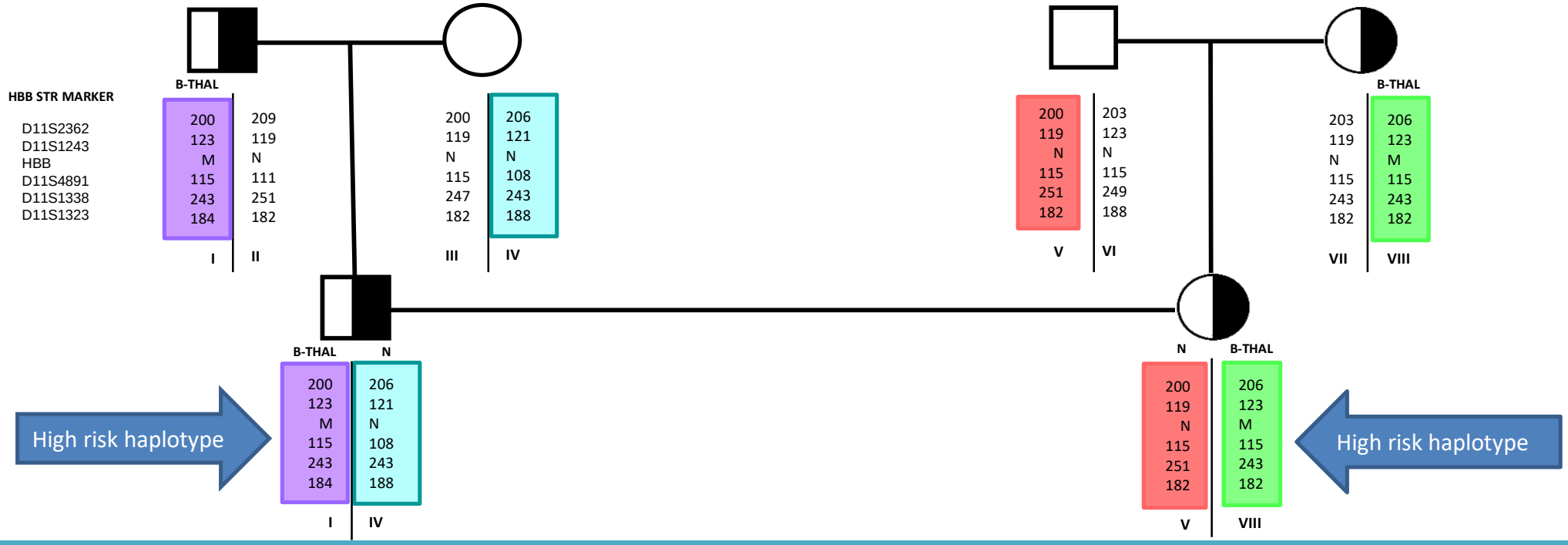
- Case: β-haemoglobinopathies
- 1st PCR: Multiplex-PCR for the amplification of the β-globin gene and 5 β-Locus STRs
- WGA can be used as an alternative method (generic)

STRs: Short tandem repeats. Highly polymorphic and abundant in human genome. Useful STRs can be taken from published papers or *in silico* selected from public databases

Preclinical Work-up

Informativity/segregation analysis

- The pathogenic variants are confirmed
- Family study for selecting the informative STR markers or SNPs
- Determination of the low-risk and high-risk haplotypes*



* Family study analysis for the determination of the high risk haplotypes is not possible for *de novo* pathogenic variants

Preclinical Work-up

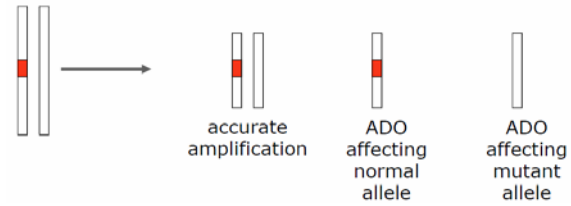
Development of multiplex 1st PCR and the 2nd genetic test

- Establishment of multiplex single cell (few cells) PCR conditions
  - ✓ Multiplex of STRs + pathogenic variant (10 ng genomic DNA)
  - ✓ Multiplex of STRs + pathogenic variant (100 pg genomic DNA)
  - ✓ Multiplex of STRs + pathogenic variant (single cell)

- PCR product from the 1st test is used for the 2nd test:
  - Targeted mutation detection
  - STR analysis

Single Cell PCR Amplification challenges

- Problems of specificity and background signals
- Amplification failure (AF)
- Allele drop out (ADO)
- Contamination
- Need for extensive optimization and validation of PCR conditions
- The inclusion of genetic markers in the clinical test allows for the detection of ADO, contamination and recombination



ADO: Allele drop out, AF Amplification failure

Preclinical Work-up

Validation

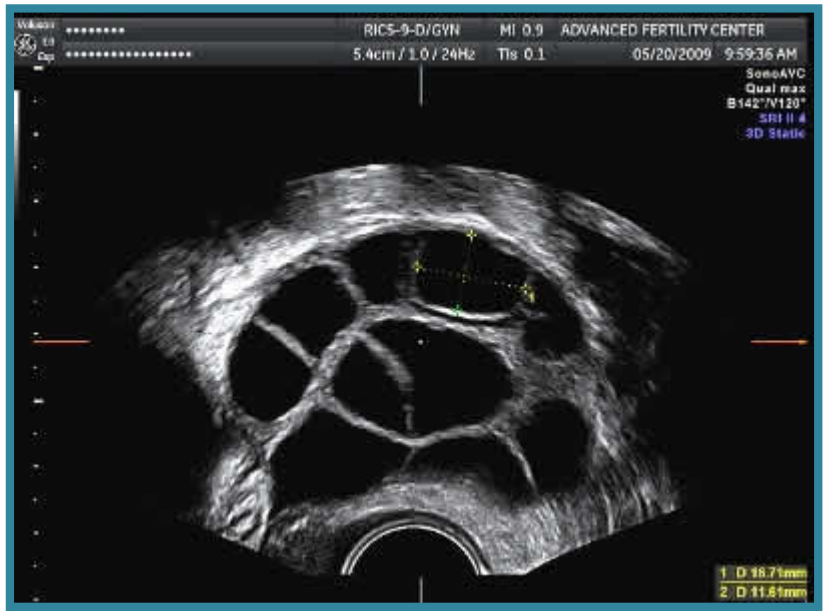
- Validation: several sets of single cells (50 lymphoblast) and controls
  - ✓ **Determination of amplification failure (AF) and ADO rate**

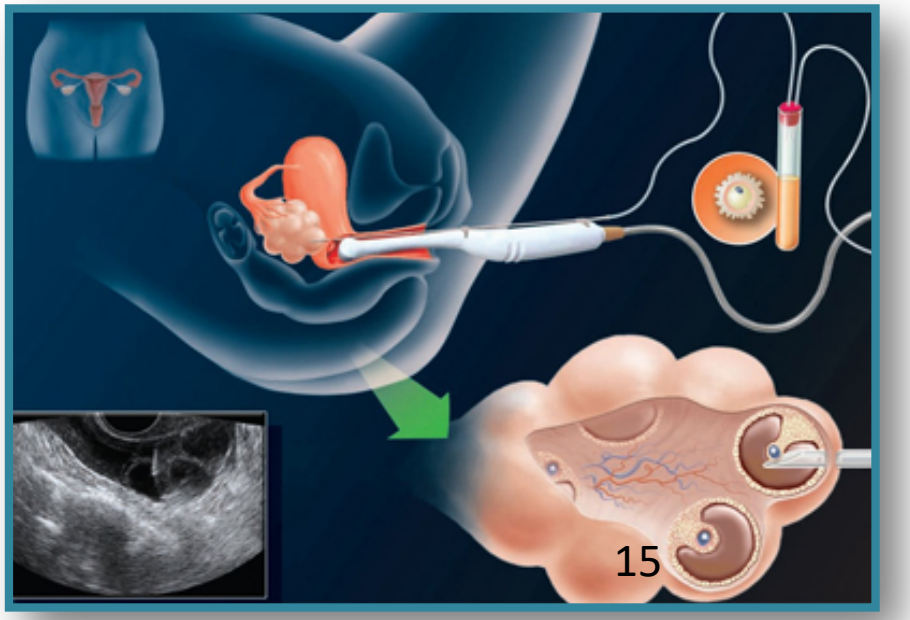
- Case: β-haemoglobinopathies
- 1st PCR: Multiplex-PCR for the amplification of the β-globin gene and 5 β-Locus STRs
- Generic test for β-haemoglobinopathies

ESHRE recommendation: ADO and AF <5%

Induction of Ovulation and Trans-vaginal Oocyte Retrieval (TVOR)

- Controlled ovarian stimulation (COS) with the use of follicle stimulating hormone (FSH) in order to ensure multiple follicle development
- Oocytes are retrieved trans-vaginally under ultrasound guidance by the obstetrician
- Oocytes and embryos are cultured using specific media and conditions and are handled by embryologists





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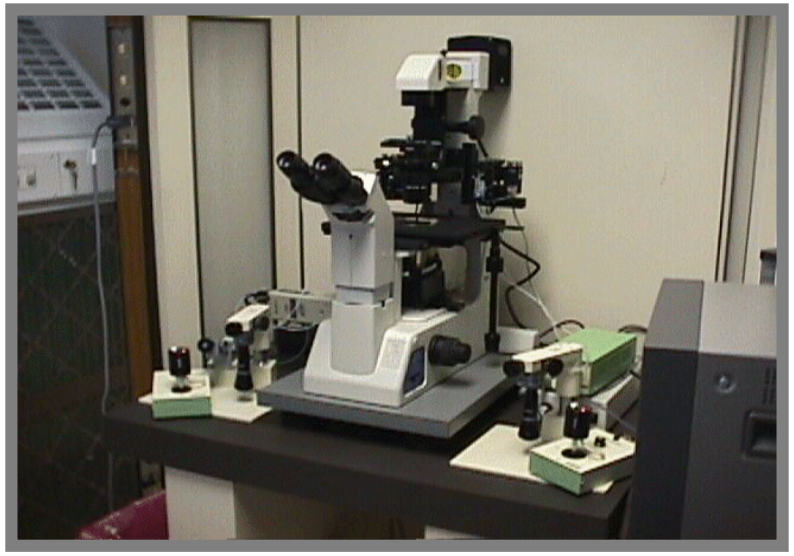
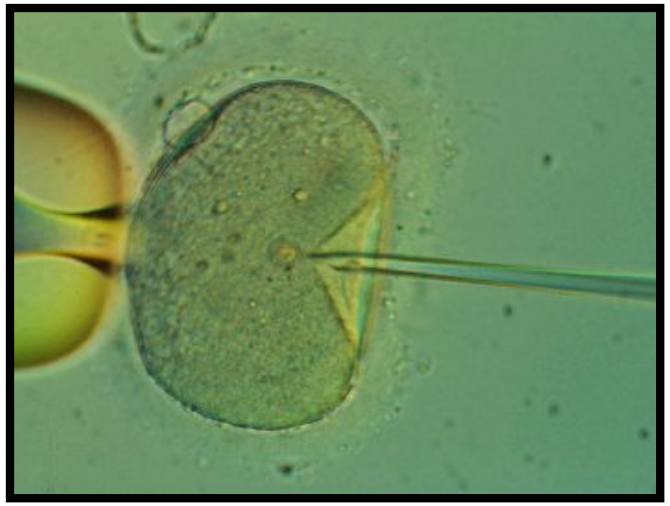
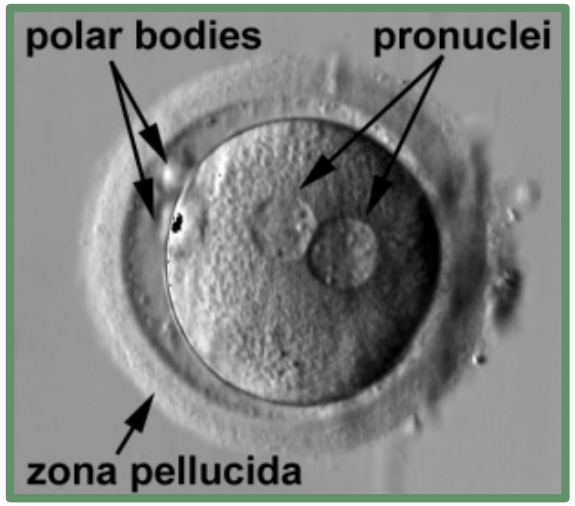
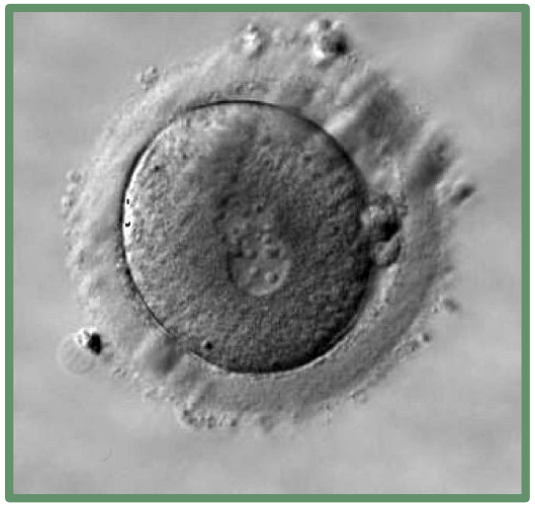
In Vitro Fertilization

- Intracytoplasmic sperm injection (ICSI)
- Second polar body extrusion and pronuclear formation
- Pronuclei fusion and zygote formation





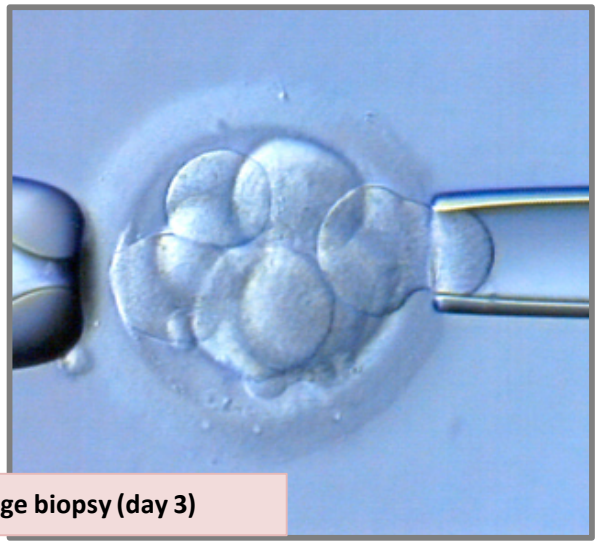




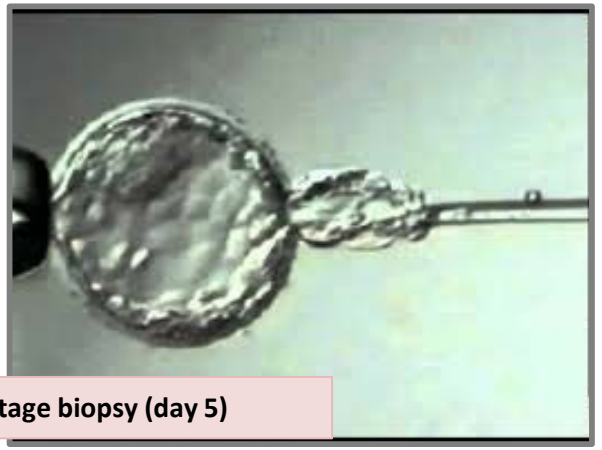
Embryo Biopsy

- Polar body biopsy
- Cleavage stage biopsy (day 3) – blastomere (1 cell)
  - The blastomere biopsy at day 3, alongside single cell multiplex PCR and fresh embryo transfer on day 5/6 has been the gold standard for over two decades
- Blastocyst stage biopsy (day 5) – trophectoderm cells (TE) (5-6 cells)
  - TE biopsy is currently the norm for embryo biopsy and is linked with the freeze-all strategy

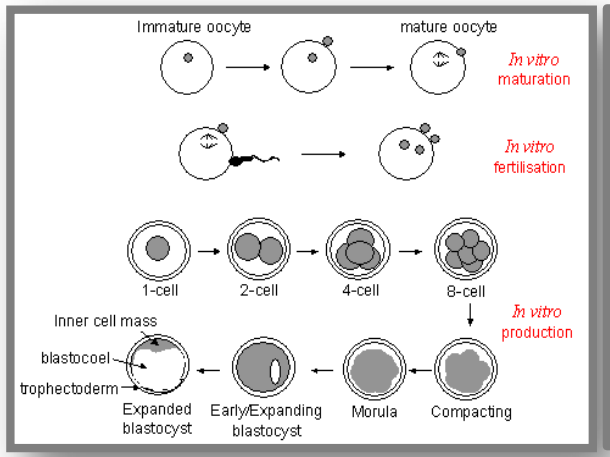
Polar body biopsy

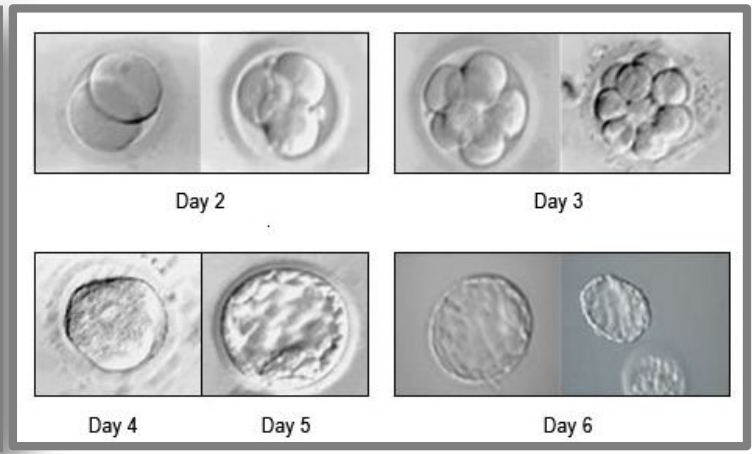


Cleavage stage biopsy (day 3)



Blastocyst stage biopsy (day 5)





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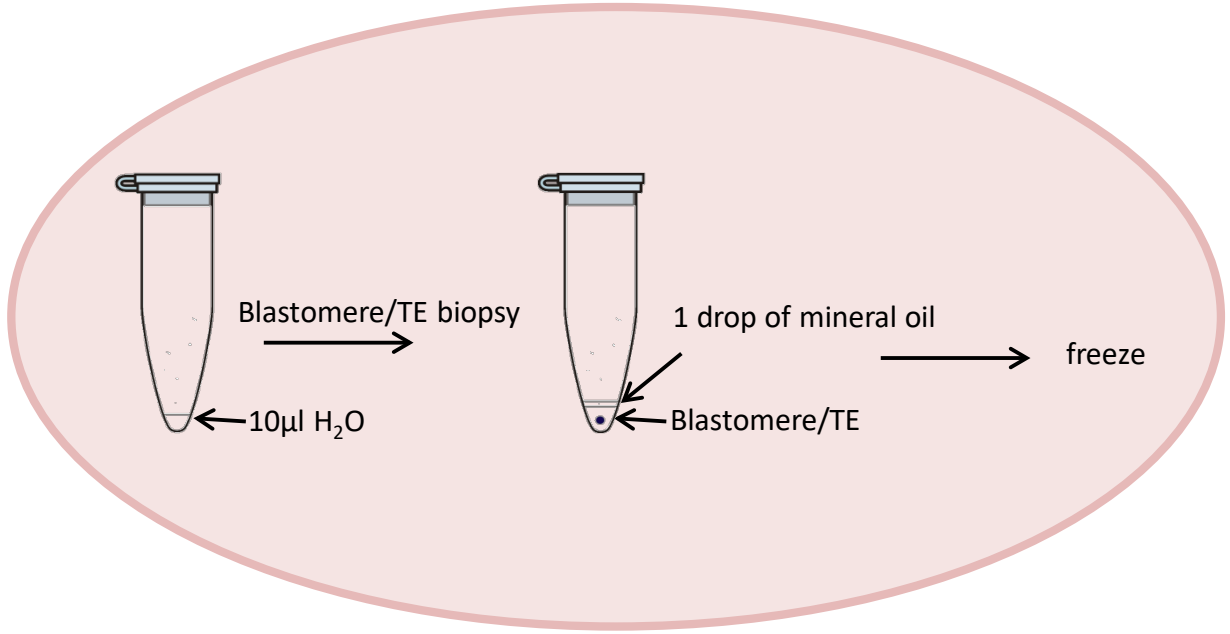
Sample Handling Protocol

- 10µl H_2O sterile distilled water is added in PCR tubes
- Biopsy is washed and added in the water
- One drop of mineral oil is added on top
- Samples are frozen
- Frozen biopsies are transferred to the Molecular Diagnostic Laboratory (PGT-M Lab)



PGT Centre

Dedicated PGT-M Laboratory

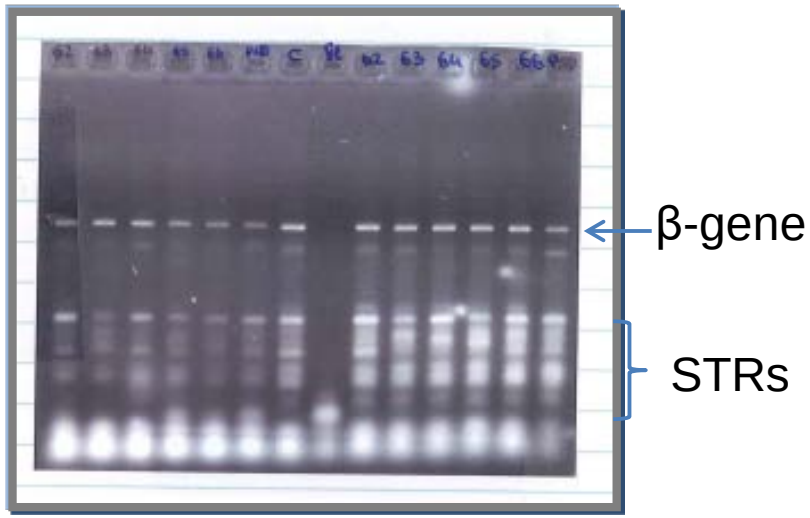
- Lab under positive pressure (avoid entry of dust and PCR products)
- DNA free area (DNA decontamination by UV)
- Laminar flow
- All work is performed by very well trained personnel

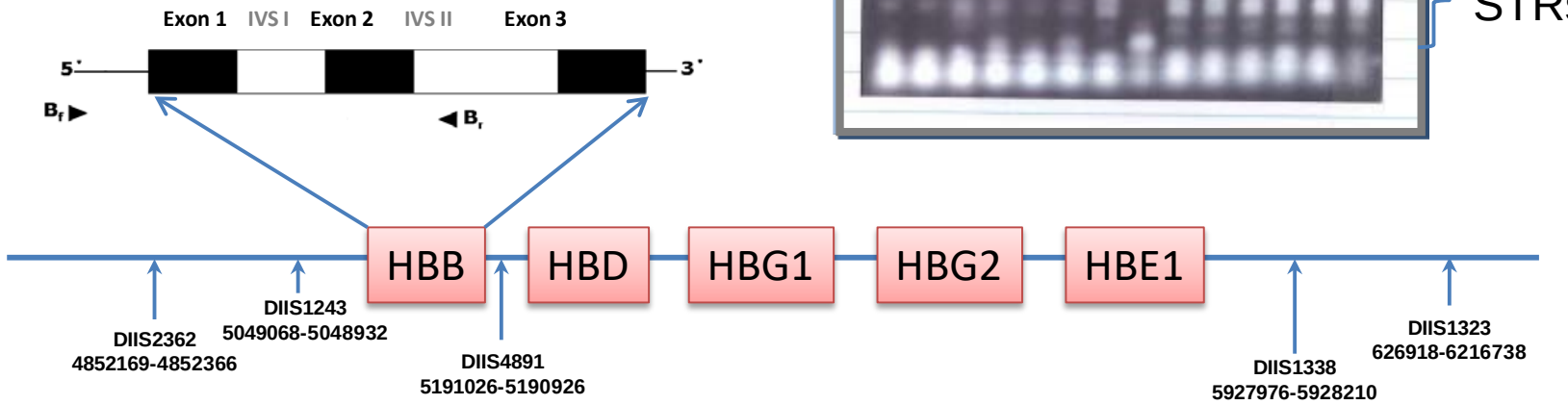
Preparation of the 1st PCR

- Proteinase-K is added to all samples for cell lysis
- PCR reagents are directly added to the lysed cell(s)
- Samples undergo targeted multiplex PCR amplification or WGA

Embryo Biopsies Analysis - 1st PCR

- Case: β-haemoglobinopathies
- 1st PCR: Multiplex-PCR for the amplification of the β-globin gene and 5 β-Locus STRs
- WGA can be used as an alternative method (generic)





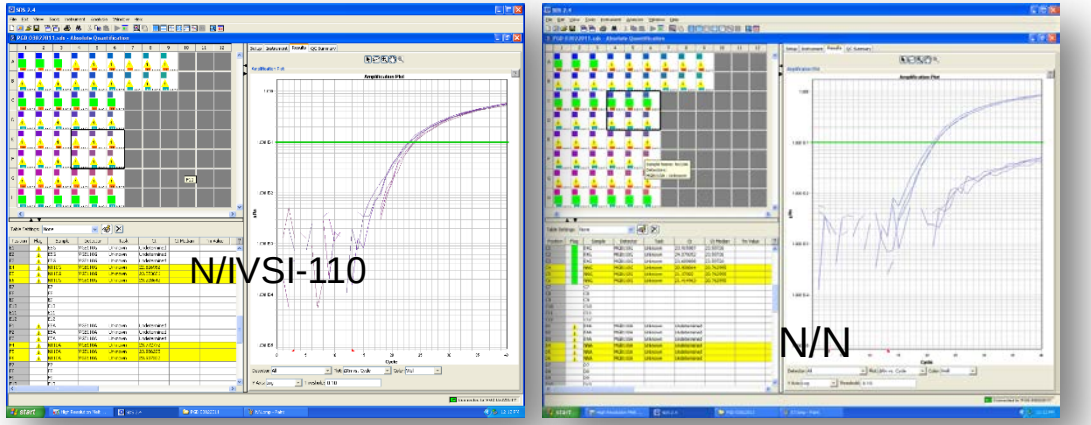
STR: Short tandem repeats, very polymorphic DNA markers
ADO: Allele drop out, one of the two alleles fails to amplify

C. Vrettou et al , (1999) Prenat. Diagn. 19: 1209 – 1216, Zachaki et al, Hemoglobin 2011;35(1):56-66, Claudia et al. Prenat. Diagn. 2009: 29: 50-56

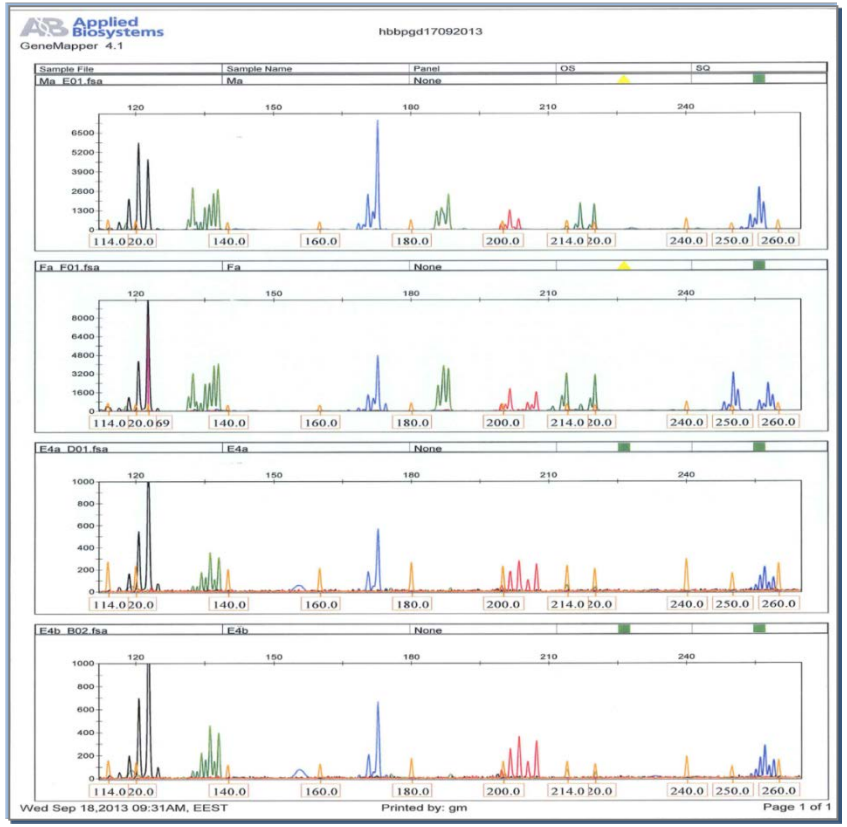
Embryo Biopsies Analysis – 2nd PCR and β-Locus STR Analysis

- 2nd PCR for mutation detection
- SNP arrays or NGS can be used as alternative methods (generic)

Real Time PCR



Fragment analysis



Mother

Father

Embryo 1A

Embryo 1B

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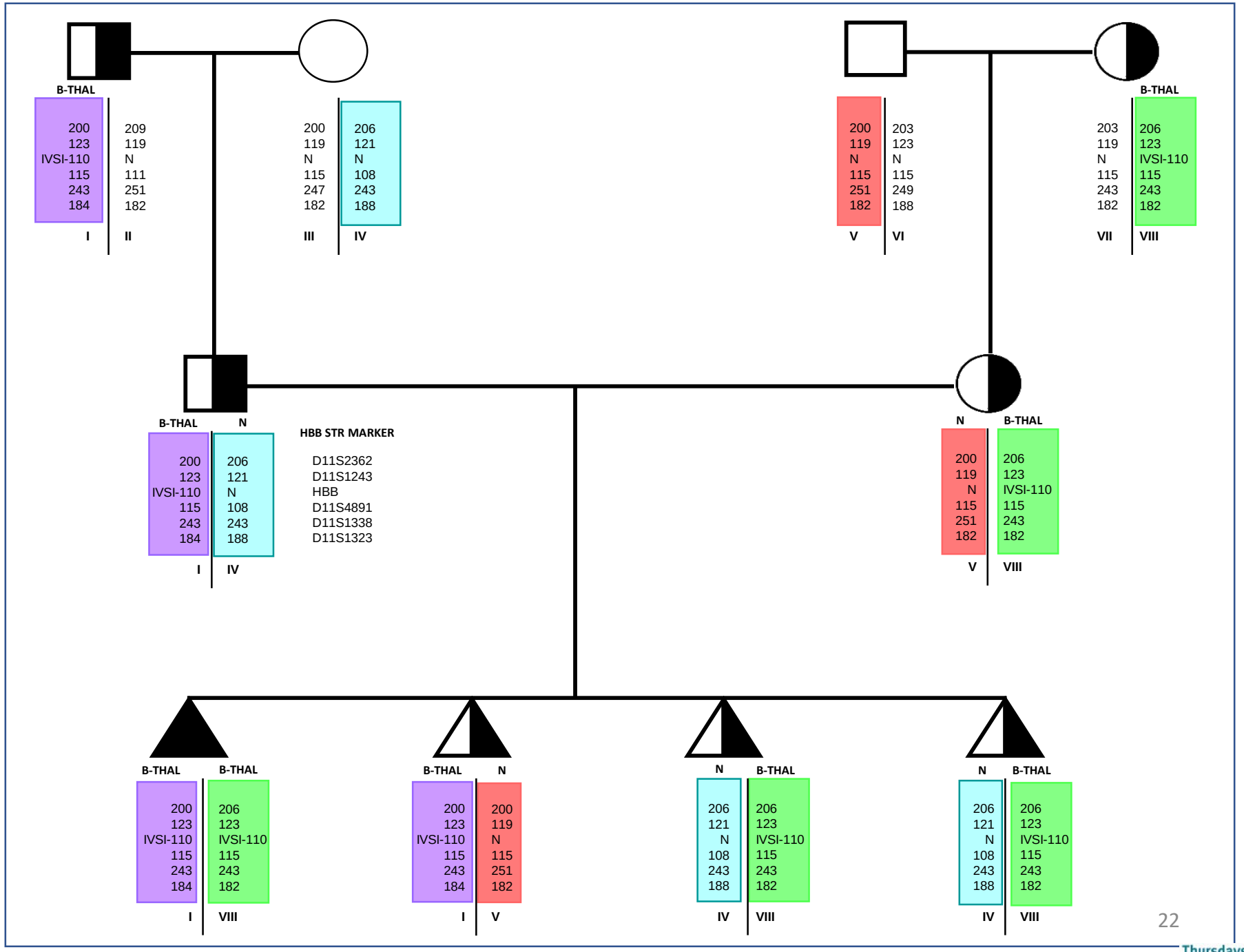
PGT-M for β-thalassaemia



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PGT-M Results

SAMPLE	GENOTYPE	DIAGNOSIS	COMMENTS
Embryo 1	IVSI-110/IVSI-6	β-THAL MAJOR	Unusable
Embryo 2	N/IVSI-110	β-THAL TRAIT	**OK for transfer**
Embryo 3	IVSI-110/IVSI-6	β-THAL MAJOR	Unusable
Embryo 4	IVS I-110/IVSI-6	β-THAL MAJOR	Unusable
Embryo 5	N/IVSI-110	β-THAL TRAIT	**OK for transfer**
Embryo 6	IVSI-110/Allelic dropout	-	Unusable
Embryo 7	N/N	NORMAL	**OK for transfer**
Embryo 8	IVS I-110/IVSI-6	β-THAL MAJOR	Unusable
Embryo 9	N/N	NORMAL	**OK for transfer**
Embryo 10	No Amplification	-	Unusable
Embryo 11	N/IVSI-6	β-THAL TRAIT	**OK for transfer**
Embryo 12	N/N	NORMAL	**OK for transfer**
Embryo 13	N/N	NORMAL	**OK for transfer**
NORMAL CONTROL		OK	
HETEROZYGOUS CONTROLS		OK	
BLANK CONTROLS		CLEAR	

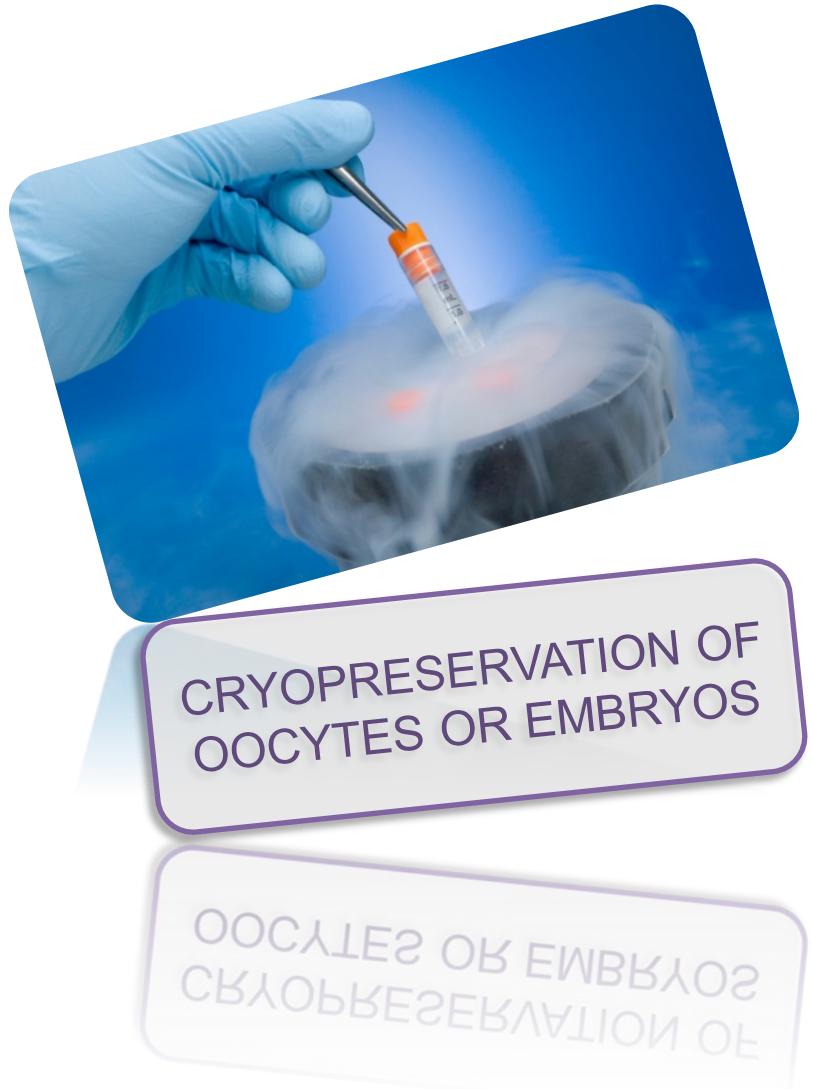
Embryo Transfer

- Transfer one embryo into the uterus
- Confirmation of pregnancy
  - ✓ Day 10 after embryo transfer (serum β-hCG assay)
  - ✓ Ultrasound at 6 weeks
- Prenatal diagnosis (ESHRE guidelines)
- Delivery

β-hCG: human chorionic gonadotropin

Cryopreservation

- Freezing and storing of extra embryos for future use
- Cryopreservation process (Vitrification)
- Stored in liquid nitrogen (-196^{0}C)



PGT - Cyprus

- The PGT-M lab was established in 2004
- It is the reference centre in Cyprus
- Provides hands on training
- 415 PGT-M tests (7 HLA typing)
- Pregnancy rate per embryo transfer is approximately 35%
- No misdiagnoses reported (ESHRE data <1%)

Accreditation process (ISO15189)	• Preimplantation Genetic Diagnosis accredited with ISO15189 by the Cyprus Accreditation body CY-CYSAB
Good practice PGT-M guidelines	• Operates according to the ESHRE good practice recommendations for PGT
External quality assessment (EQA)	• UK National External Quality Assessment Service (UK NEQAS) (http://www.ukneqas-molgen.org.uk))

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CYPRUS PGT-M RESULTS 2004 – 2020

25% of the couples at risk for haemoglobinopathies in Cyprus use PGT-M as an alternative to PD

Monogenic Disorder	No PGD tests
β-thalassaemia	352
Rare β-haemoglobinopathies (SCD, HbKnossos, HbLepore, HbC etc.)	15
β-haemoglobinopathies combined with HLA matching	7
β-haemoglobinopathy combined with Autosomal Dominant Nephropathy	1
α-thalassaemia (Hb Barts Hydrops foetalis)	4
Tay-Sachs Disease (TSD)	1
Autosomal Dominant SDHB Paraganglioma	3
Huntington's Disease (HD)	3
Charcot-Marie-Tooth 1A (CMT1A)	2
Cystic Fibrosis (CF)	4
Congenital Deafness (Connexin26)	1
Multiple Endocrine Neoplasia 2B (MEN2B)	2
Duchenne/Becker Muscular Dystrophy	1
Cerebral Cavernous Malformations (CCM1)	2
Hypochondroplasia (FGFR3)	2
Myotonic Dystrophy (DM)	1
Multiple Endocrine Neoplasia Type2A (MEN2A)	1
Incontinentia Pigmenti (IP)	2
Léri-Weill Dyschondrosteosis (SHOX)	1
Cowden Syndrome	2
Prion Disease	6
Spinal Muscular Atrophy	1
Familial Hypercholesterolaemia (FH)	1
Friedreich Ataxia (set-up awaiting biopsies)	
Total	415

Rare Anaemias (EuroBloodNet list)

HH and iron	Rare anaemia	Sideroblastic anaemias	Constitutional sideroblastic anaemia	
HH and iron	Rare anaemia	Rare deficiency anaemia	Constitutional deficiency anaemia	Constitutional anaemia due to iron metabolism disorder
HH and iron	Rare anaemia	Rare hereditary hemochromatosis		
HH and iron	Porphyrias			
RBC defects	Haemoglobinopathies			
RBC defects	Rare anaemia	Rare haemolytic anaemia	Rare constitutional haemolytic anaemia	Rare constitutional haemolytic anaemia due to RBC membrane anomaly
RBC defects	Rare anaemia	Rare haemolytic anaemia	Rare constitutional haemolytic anaemia	Rare constitutional haemolytic anaemia due to an enzyme disorder
Bleeding	Rare anaemia	Rare haemolytic anaemia	Rare constitutional haemolytic anaemia	Atypical haemolytic-uremic syndrome
BMF	Rare anaemia	Aplastic anaemia	Rare constitutional aplastic anaemia	
BMF	Rare anaemia	Rare deficiency anaemia	Constitutional deficiency anaemia	
BMF	Rare anaemia	Constitutional dyserythropoietic anaemia		

PGT-M for Rare Anaemias

Rare anaemias

- In all groups of rare anaemias high risk and serious diseases are included
- The genetic diagnosis is not known for many rare anaemias
- NGS and functional studies may be needed in order to identify the causative pathogenic variant
- BMT is a curative treatment for haematological diseases

PGT-M for rare anaemias

- PGT-M can be used for the **high risk and serious rare anaemias** for which the disease-causing loci have been unequivocally identified
  - Nuclear (X-linked, autosomal, dominantly or recessively inherited)
  - Mitochondrial (maternally inherited) and involve (likely) pathogenic genetic variant(s)
- HLA typing with or without concurrent testing for a monogenic disorder

PGT-M - Special Cases

- De novo pathogenic variants
- Consanguineous families
- **HLA typing**
- Exclusion testing (e.g. Huntington disease)
- Mitochondrial DNA disorders

HLA Typing with Concurrent Testing for a Monogenic Disorder

- BMT is the only curative treatment for β-thalassaemia, SCD and other haematological diseases
- HLA typing can be provided for selecting and transferring unaffected embryos who are HLA-identical with an affected sibling
- HSC from the ‘savior’ child are transplanted to the affected sibling
- Haematopoietic reconstruction was successful in almost all reported cases

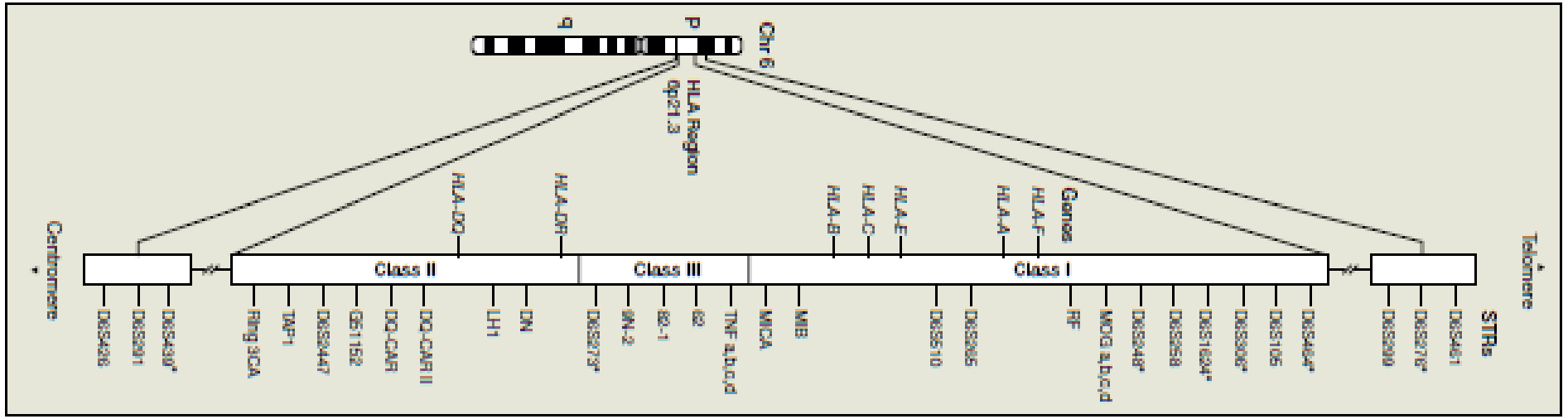
BMT: Bone marrow transplantation

HLA: Human Leucocyte Antigens

HSC: Haematopoietic stem cells

PGT-M for β-Haemoglobinopathies and HLA Typing

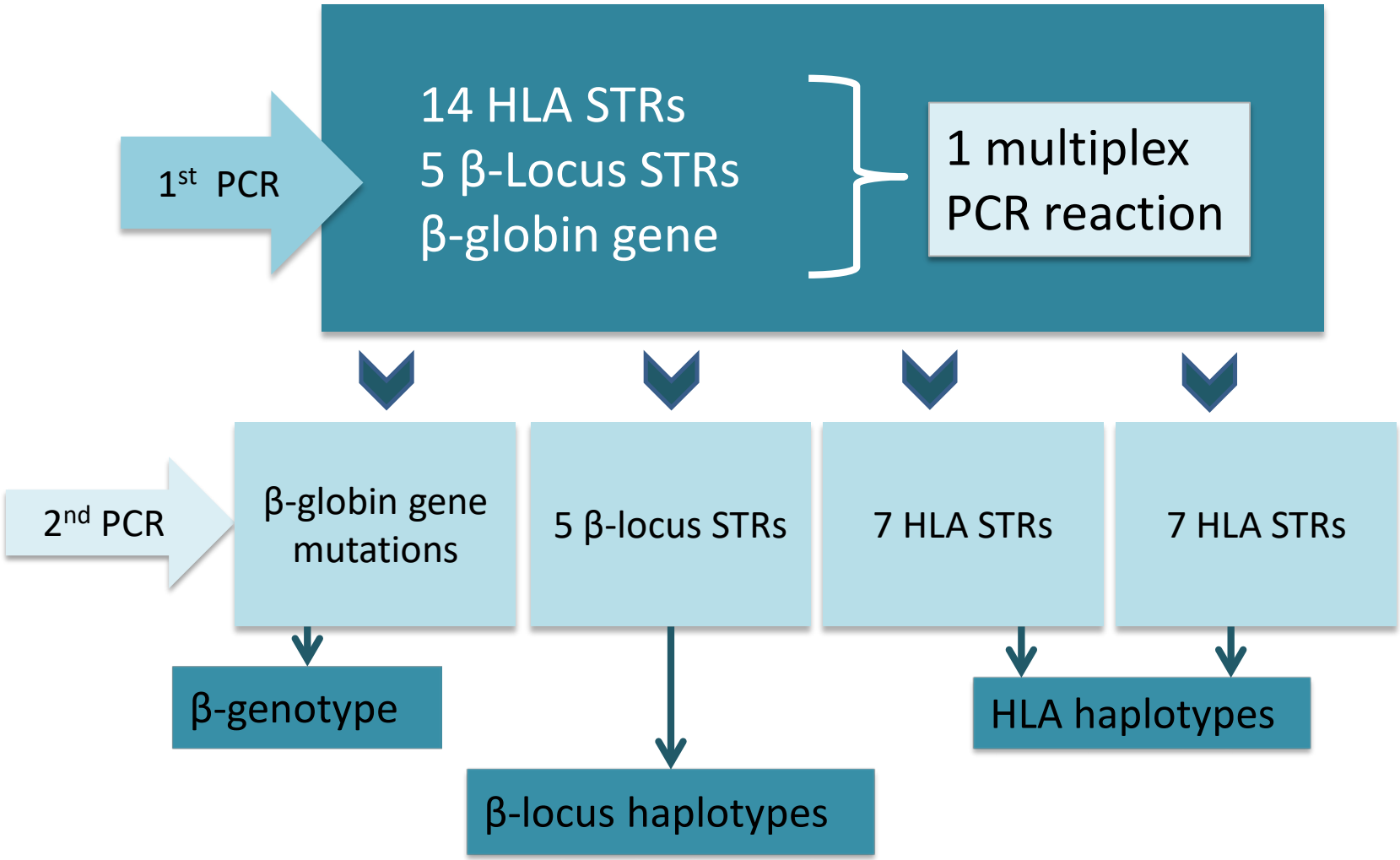
14 STRs selected to span the whole MHC region and linked to class I, class II and class III HLA genes

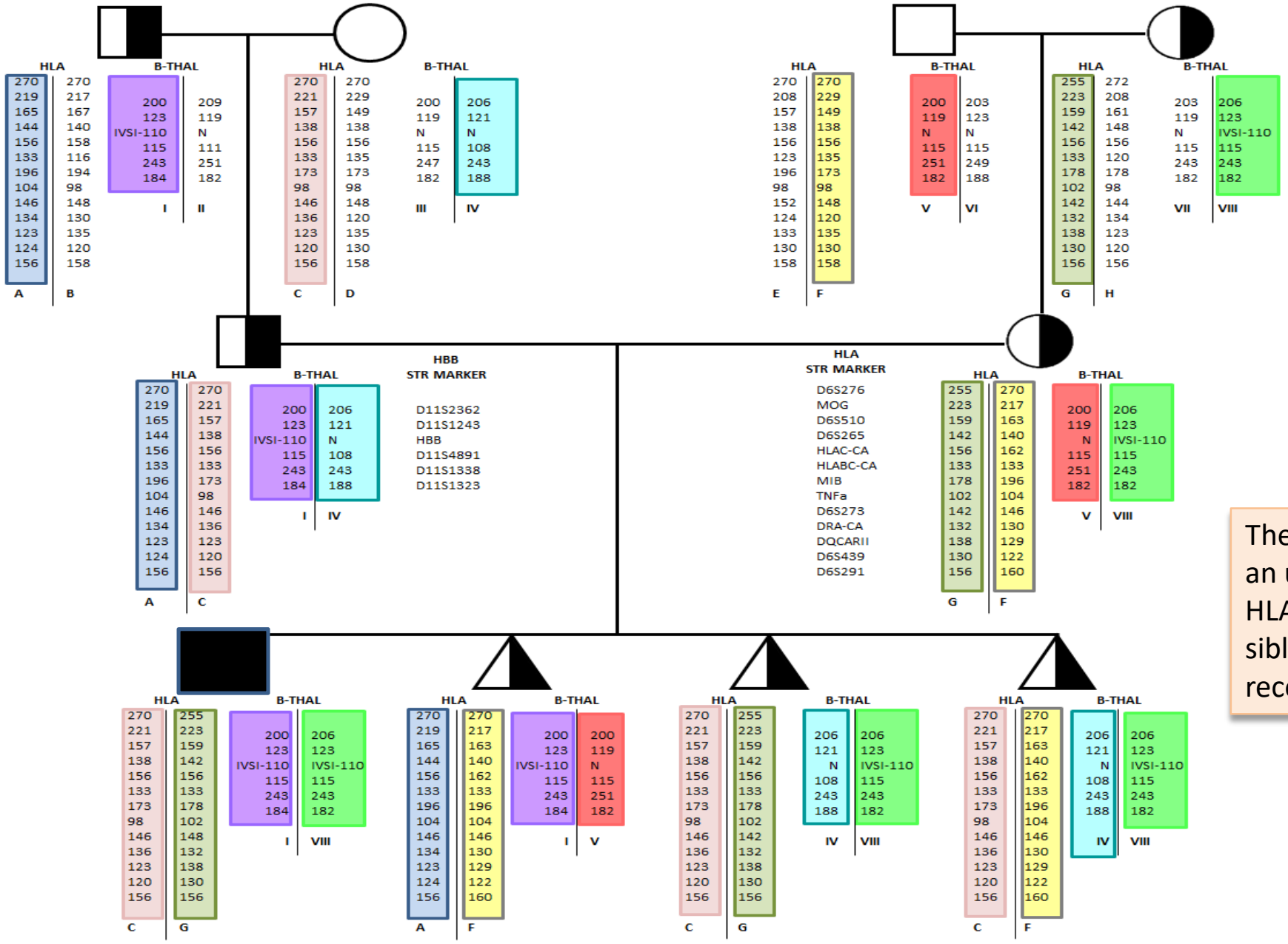


MHC: Major Histocompatibility Complex

Fiorentino et al, European Journal of Human Genetics. 2005;13: 953-958, Fiorentino et al, Molecular Human Reproduction. 2004;10: 445-460, Van de Velde et al, Human Reproduction. 2004;19: 700-708

OUR APPROACH





The probability of identifying an unaffected embryo who is HLA-identical with an affected sibling is 3 out of 16 embryos in recessive diseases

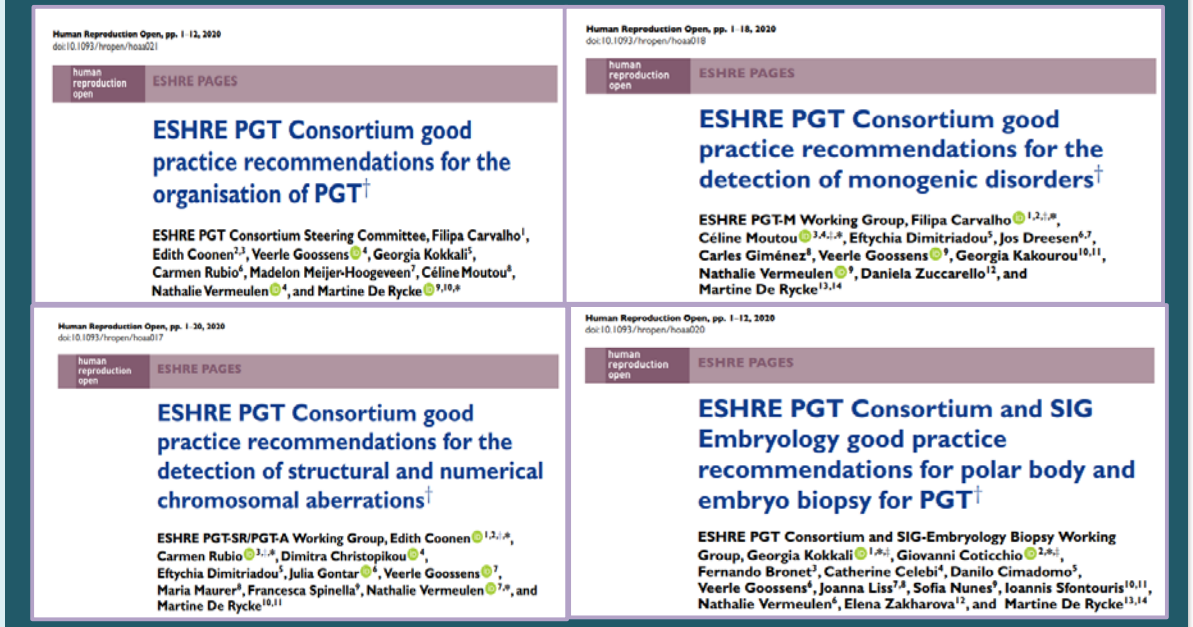
PGT-M Stages

PGT Cycle

- Patient inclusion/exclusion
- Counseling – informed consent
- Preclinical work-up
- Induction of ovulation
- Oocyte collection
- Fertilization by ICSI
- Embryo culture and biopsy
- Genetic testing
- Implantation of one suitable embryo
- Confirmation of pregnancy
- Post-examination process

Other Aspects

- Basic requirements of a PGT centre
- Transport PGT
- Accreditation and quality management
- Follow-up of PGT pregnancies and children



Human Reproduction Open, pp. 1–12, 2020
doi:10.1093/hropen/hoaa021
human reproduction open — ESHRE PAGES

ESHRE PGT Consortium good practice recommendations for the organisation of PGT†

ESHRE PGT Consortium Steering Committee, Filipa Carvalho[1], Edith Coonen[2,3], Veerle Goossens[4], Georgia Kokkali[5], Carmen Rubio[6], Madelon Meijer-Hoogeveen[7], Céline Moutou[8], Nathalie Vermeulen[4], and Martine De Rycke[9,10,*]

Human Reproduction Open, pp. 1–18, 2020
doi:10.1093/hropen/hoaa018
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ESHRE PGT Consortium good practice recommendations for the detection of monogenic disorders†

ESHRE PGT-M Working Group, Filipa Carvalho[1,2,‡,*], Céline Moutou[3,4,‡,*], Eftychia Dimitriadou[5], Jos Dreesen[6,7], Carles Giménez[8], Veerle Goossens[9], Georgia Kakourou[10,11], Nathalie Vermeulen[9], Daniela Zuccarello[12], and Martine De Rycke[13,14]

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ESHRE PGT Consortium good practice recommendations for the detection of structural and numerical chromosomal aberrations†

ESHRE PGT-SR/PGT-A Working Group, Edith Coonen[1,2,‡,*], Carmen Rubio[3,‡,*], Dimitra Christopikou[4], Eftychia Dimitriadou[5], Julia Gontar[4], Veerle Goossens[7], Maria Maurer[8], Francesca Spinella[9], Nathalie Vermeulen[7,*], and Martine De Rycke[10,11]

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ESHRE PGT Consortium and SIG Embryology good practice recommendations for polar body and embryo biopsy for PGT†

ESHRE PGT Consortium and SIG-Embryology Biopsy Working Group, Georgia Kokkali[1,*,‡], Giovanni Coticchio[2,*,‡], Fernando Bronet[3], Catherine Celebi[4], Danilo Cimadomo[5], Veerle Goossens[4], Joanna Liss[7,8], Sofia Nunes[9], Ioannis Sfontouris[10,11], Nathalie Vermeulen[6], Elena Zakharova[12], and Martine De Rycke[13,14]

Take home messages

1. PGT-M is technically a very complex and demanding method
2. Established prevention alternative to prenatal diagnosis (PD) for an ever increasing number of different conditions including rare anaemias
3. PGT can be also used for the selection and transfer of unaffected embryos who are HLA-identical with an affected sibling – HSC from the ‘savior’ child are transplanted to the affected sibling

THANK YOU

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European Reference Network
for rare or low prevalence complex diseases
Network Hematological Diseases (ERN EuroBloodNet)

Thursdays Webinars