

Thalassemia Prevention : Screening and Prenatal Diagnostic Approaches

Distance Learning Course

From Research to practice: Training course in
Sexual and Reproductive Health Research

Community Genetics



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The Cyprus Institute of Neurology & Genetics

Thalassaemia

- This presentation includes:
 - Introduction
 - Thalassaemia control programs
 - Strategy for the prevention of the disease
 - Prenatal diagnostic approaches



Haemoglobinopathies

- Structure of globin chain
- Rate of synthesis of globin chains (Thalassaemias)
- Hereditary Persistence of Fetal Haemoglobin (HPFH)



Haemoglobinopathies

Thalassaemias (350)

α -thalassaemia

β -thalassaemia

Abnormal Haemoglobins (887)

Hb S

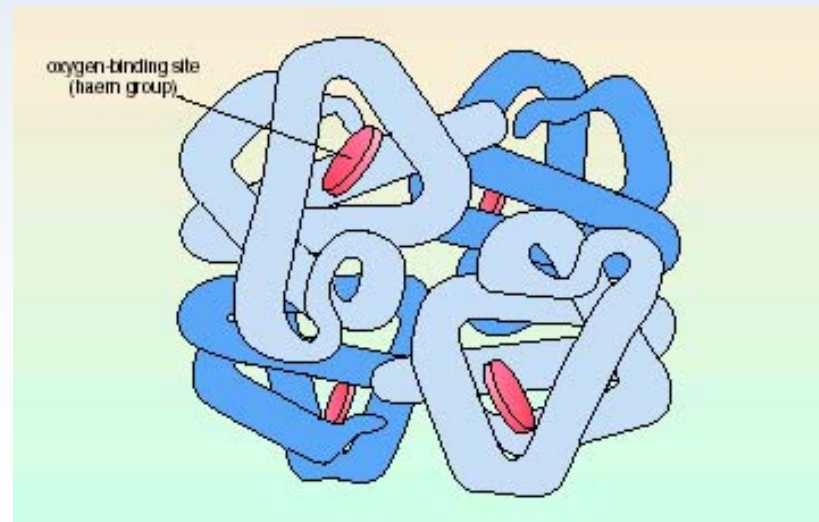
Hb D

Hb E



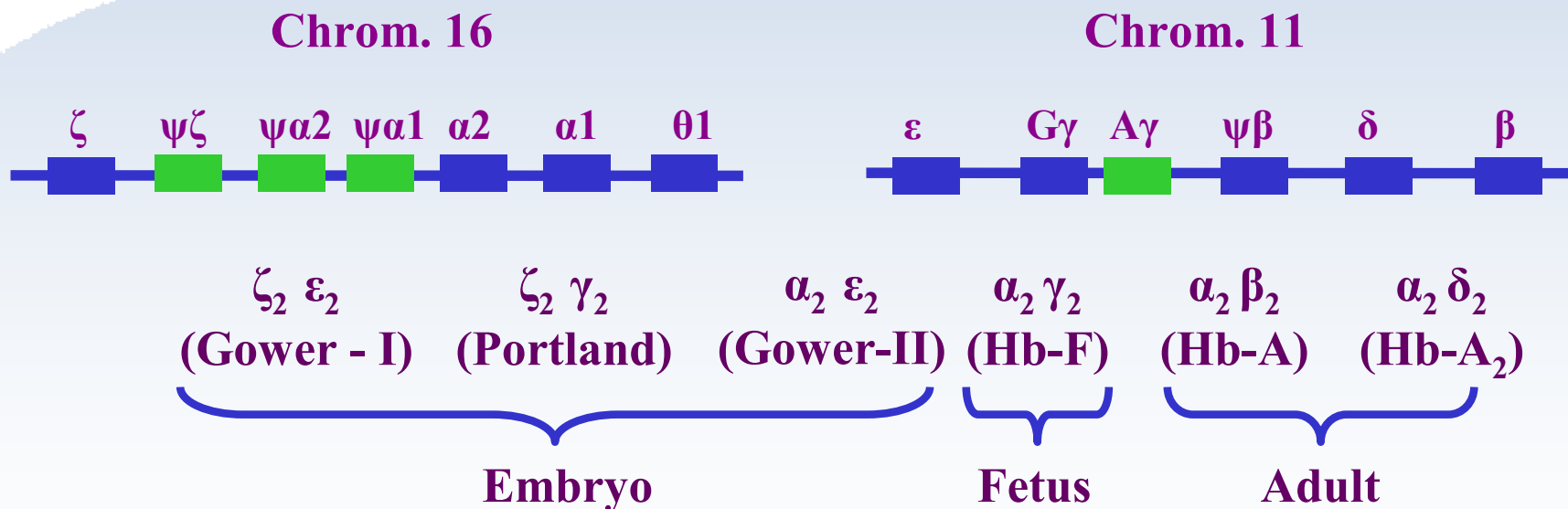
Thalassaemia

- Reduction or absence of one of the globin polypeptides making up haemoglobin
- Haemoglobin is a tetramer composed of 2 α -type globin chains and 2 β -type globin chains

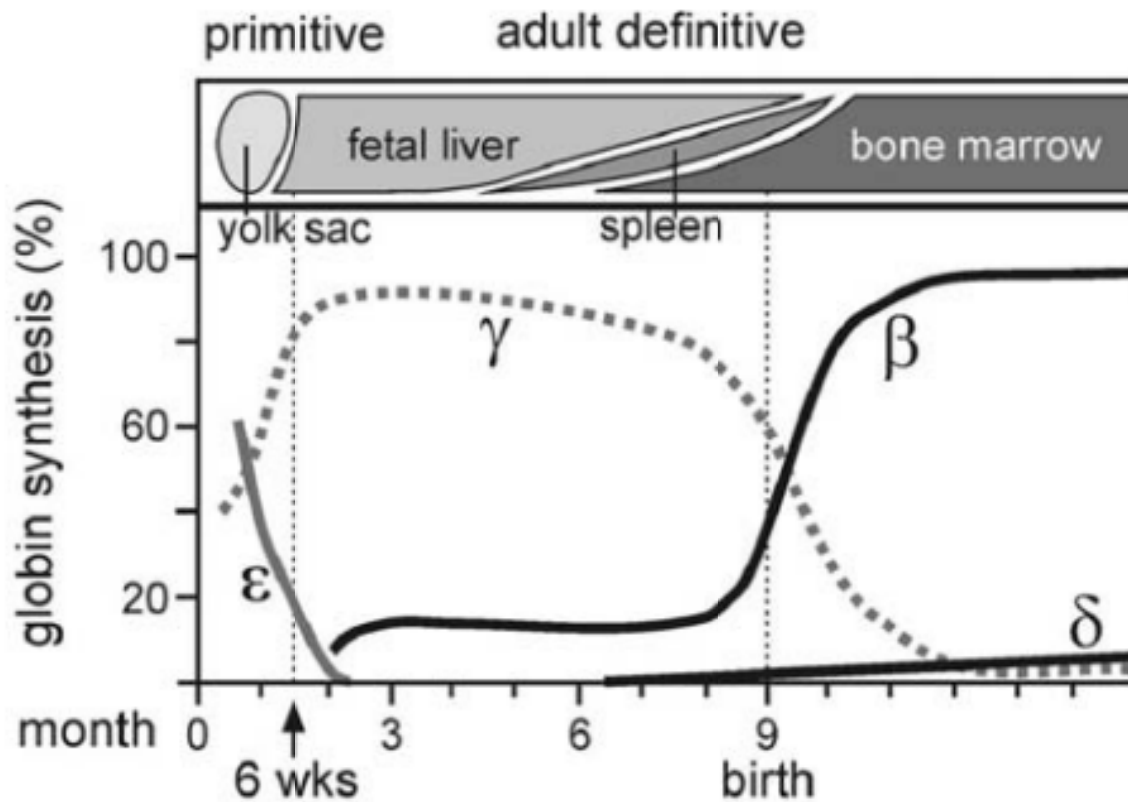


Human Haemoglobins and Globin Genes

- Thalassaemias are hereditary blood disorders caused by a reduced synthesis of one or more of the globin chains



Haematopoiesis



Embryonic haemoglobins

Gower-I $\zeta_2\epsilon_2$

Gower-II $\alpha_2\epsilon_2$

Portland $\gamma_2\zeta_2$

Foetal Haemoglobin

Hb-F $\alpha_2\gamma_2$

Adult haemoglobin

Hb-A $\alpha_2\beta_2$

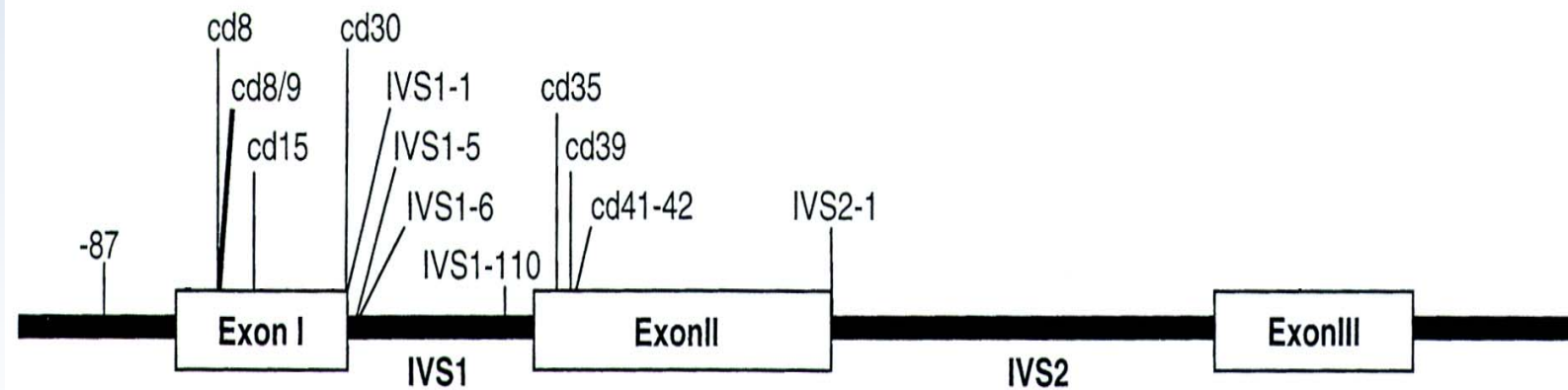
Hb-A₂ $\alpha_2\delta_2$



β -thalassaemia

>200 β -globin gene mutations

Common β -globin gene mutations

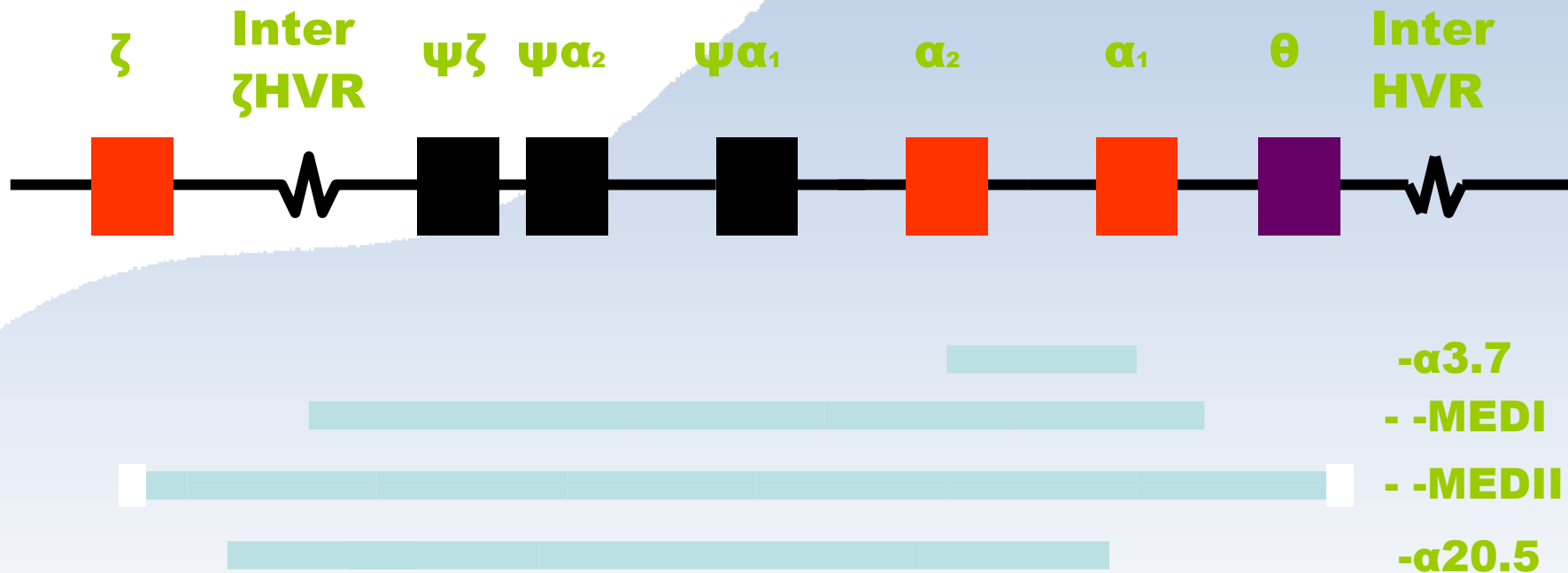


β -thalassaemia

- Globin chain imbalance
- Accumulation of excess α -globin chains in erythroid precursors (ineffective erythropoiesis) and RBC (haemolytic anaemia)



Common Deletional and non-Deletional α -Thalassaemia Mutations



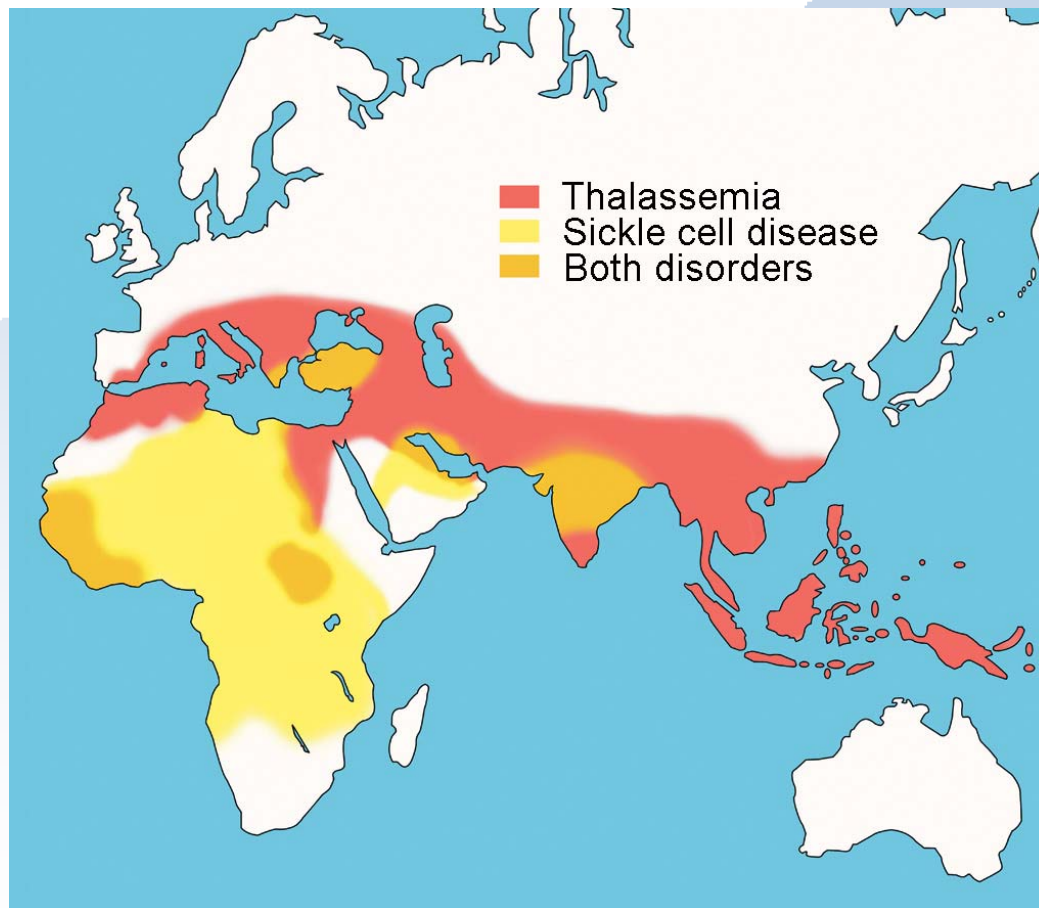
Non deletional α -thal mutations

$\alpha 2$ IVSI Donor site **GA[GGTGA]GG \rightarrow GAGG....(5nt deletion)**

$\alpha 2$ Poly(A) signal **AATAAA \rightarrow AATGAA (PA-2)**



World Distribution of Haemoglobinopathies

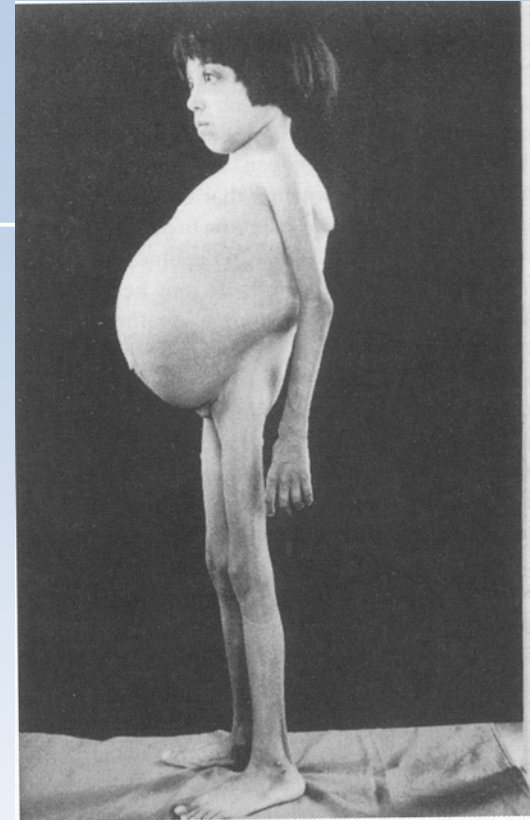


- One of the most common inherited blood disorder in the world
- 250 million people (4.5%) are carriers of a potentially pathologic gene
- 300,000 infants are born with a major haemoglobinopathy



Thalassaemia

- Severe anaemia
- Regular blood transfusion
- Iron chelation therapy
- Bone marrow transplantation (BMT)
- Gene therapy
- Drug therapy



Thalassaemia control programs

- National program - effective strategy
- Infrastructure
- Patient Treatment
- Prevention of the disease



Thalassaemia control programs

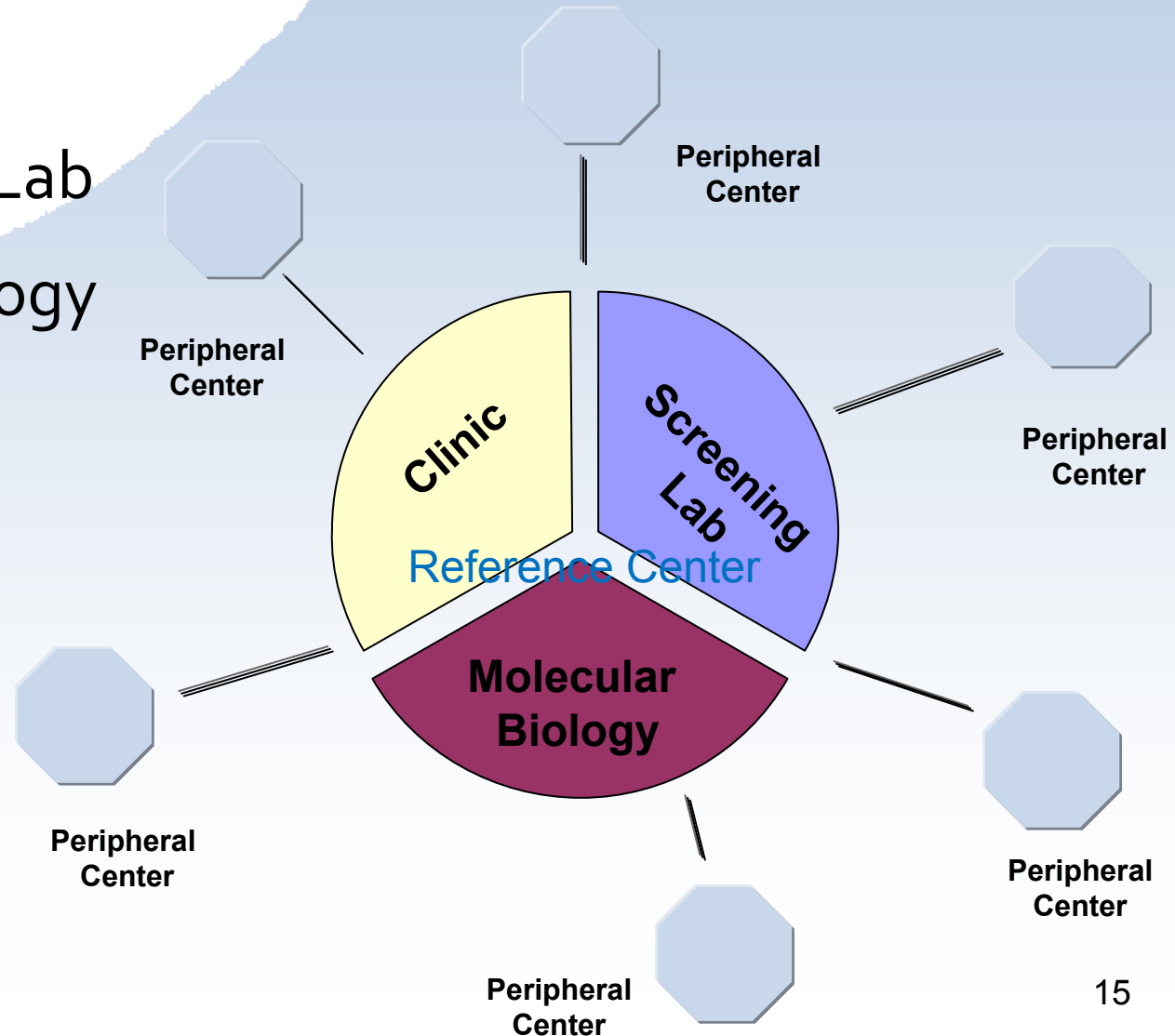
National Program – Effective Strategy

- Help from WHO and TIF and experts in the field
- Extend of the problem
- Community priorities
- Economic situation
- Distribution
- Ethical (therapeutic abortion option)
- National financial support of the program



Thalassaemia control programs Infrastructure – Thalassaemia Center

- Clinics
- Haematology Lab
- Molecular Biology Lab



Thalassaemia control programs

Prevention

- Public education
- Carrier screening
- Genetic counseling
- Prenatal Diagnosis



Prevention Programs

- Euro Mediterranean countries (Italy, Greece, Cyprus)
- Middle East countries (Iran 1997)
- SE-Asia countries (Asian Network for the control of thalassaemia was established on 2004)



Prevention Programs

Public education

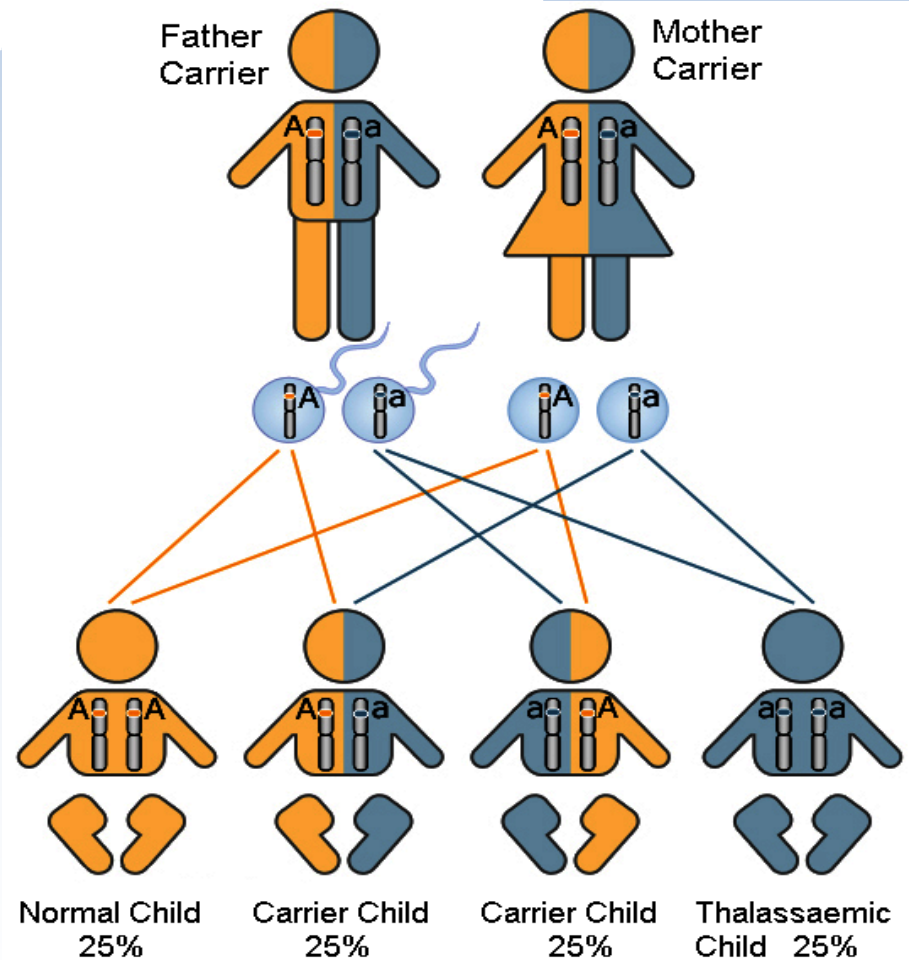
- Schools
- Leaflets
- Media
- Conferences/Seminars
- Professionals
- **To inform NOT to stigmatize**



Prevention Programs

Carrier Screening

- Population screening
- High risk groups
- Pregnant women



Carrier Detection

- Haematology
- Hbs electrophoresis
- Biosynthesis
- Family study
- Molecular diagnosis

	NORMAL	β-thal carrier
MCH (pgt)	>27	<27
HbA2(%)	<3.3	>3.5
Hb	A+A2	A+(F)+A2



Thalassaemia Carrier Screening Flow Chart

MCH (pg) **>27**
 HbA2(%) **<3.3**
 Hb **A+A2**

NORMAL

<27
>3.5
A+(F)+A2

β-THAL

SCREENING FOR COMMON β-THAL MUTATIONS
 ↓
UNDEFINED β-THAL MUTATION DGGE
 ↓
DIRECT SEQUENCING

<27
<3.5
A+A2

IRON STUDIES
α-GLOBIN GENE BY PCR
 ↓ ↓
α-THAL **NORMAL α-GENES**
 ↓
GLOBIN CHAIN SYNTHESIS AND/OR δ-GENE ANALYSIS
 ↓
δ+β-THAL, γδβ THAL
OTHER NORMAL HbA2 β-THAL

<27
<3
A+F+A2

↓
HbF
 ↓
α / β RATIO ANALYSIS
 ↓
δβ-THAL
HPFH



Prevention Programs

Genetic counseling

- Risks
- Clinical features
- Patient treatment
- Options
- Procedures to follow



Prevention Programs

Prenatal Diagnosis

- Blood samples from family members
- CVS biopsy/Amniocentesis
- Molecular analysis (ARMS, Sequencing etc)
- Diagnosis

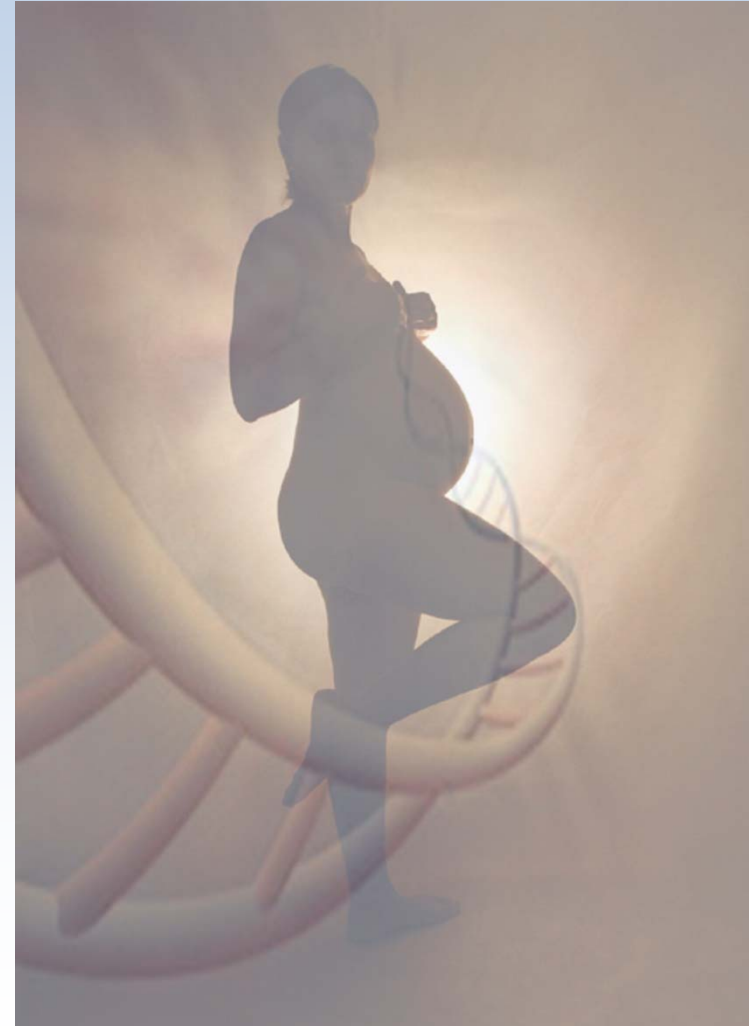


Prenatal Diagnosis

Cyprus example

- Amniocentesis (2nd Trimester)
- CVS (1st Trimester)
- PGD (Pre Implantation)

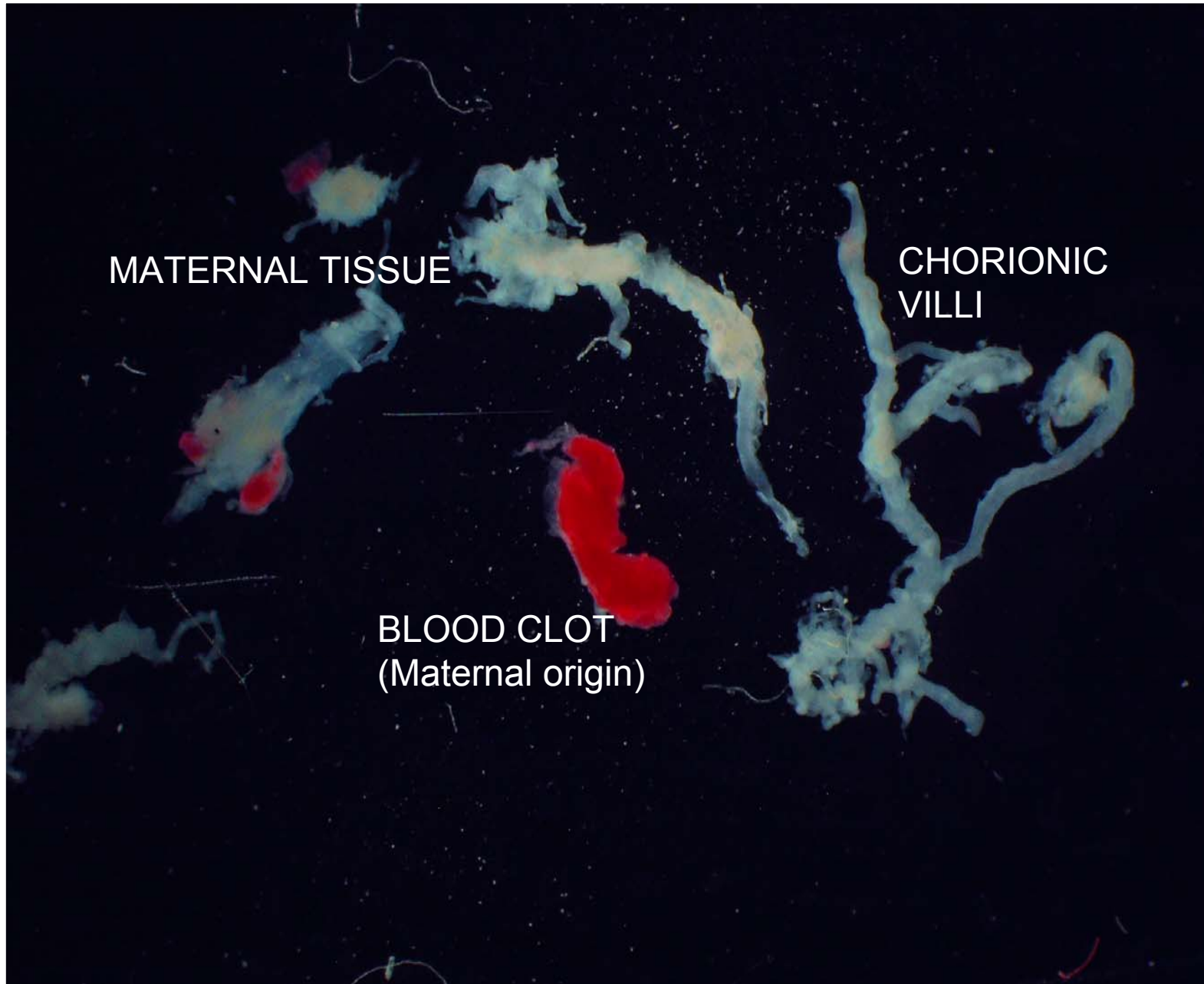
- Non invasive prenatal diagnosis
(EC FP6 Network of Excellence "SAFE")



Prenatal Diagnosis for haemoglobinopathies

- β -thalassaemia/Hb variants
- Hydrops Fetalis (α -thalassaemia)
- Severe haemoglobin H disease





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-
-
-
-
-
-
-
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-



Steps followed up for prenatal diagnosis by CVS - Cyprus experience

- Thalassaemia trait testing
- Card and Premarital certificate
- Genetic counseling
- **Pregnancy**
- Blood samples and family tree

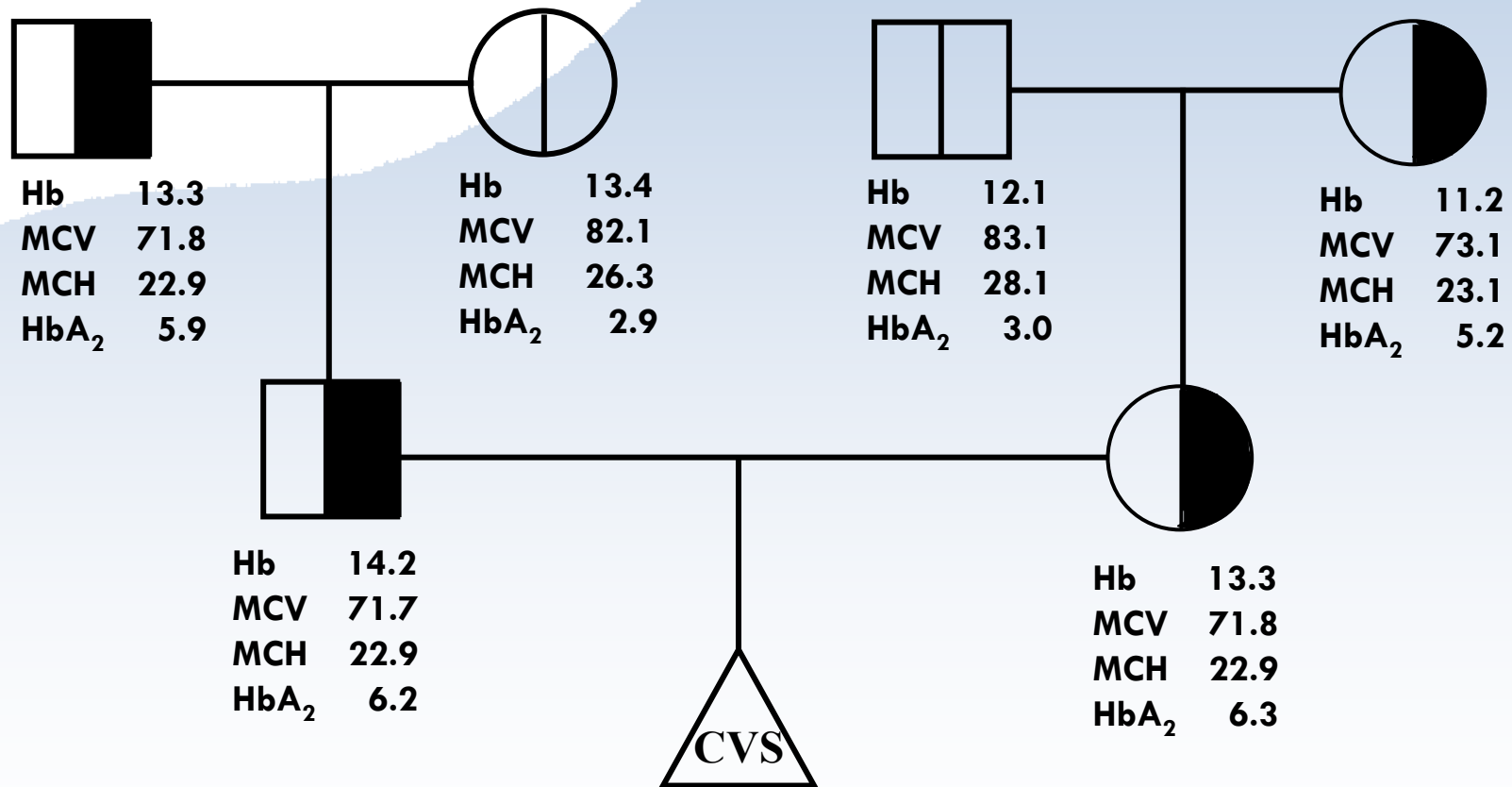
- DNA extraction
- DNA analysis of family members

- Ultrasound
- CVS biopsy at 11th week of gestation

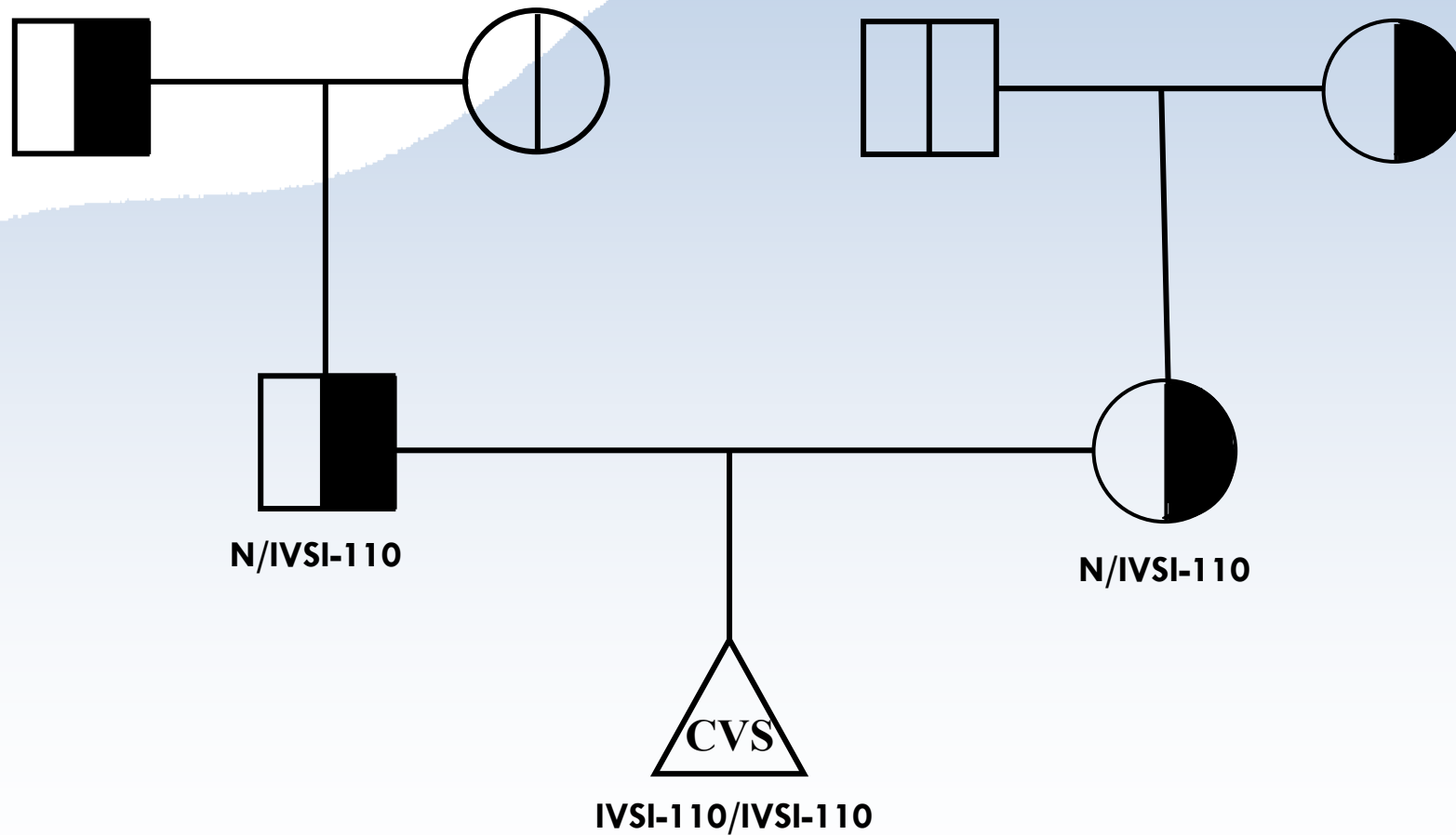
- CVS cleaning under microscope
- DNA extraction
- Molecular analysis
- Diagnosis



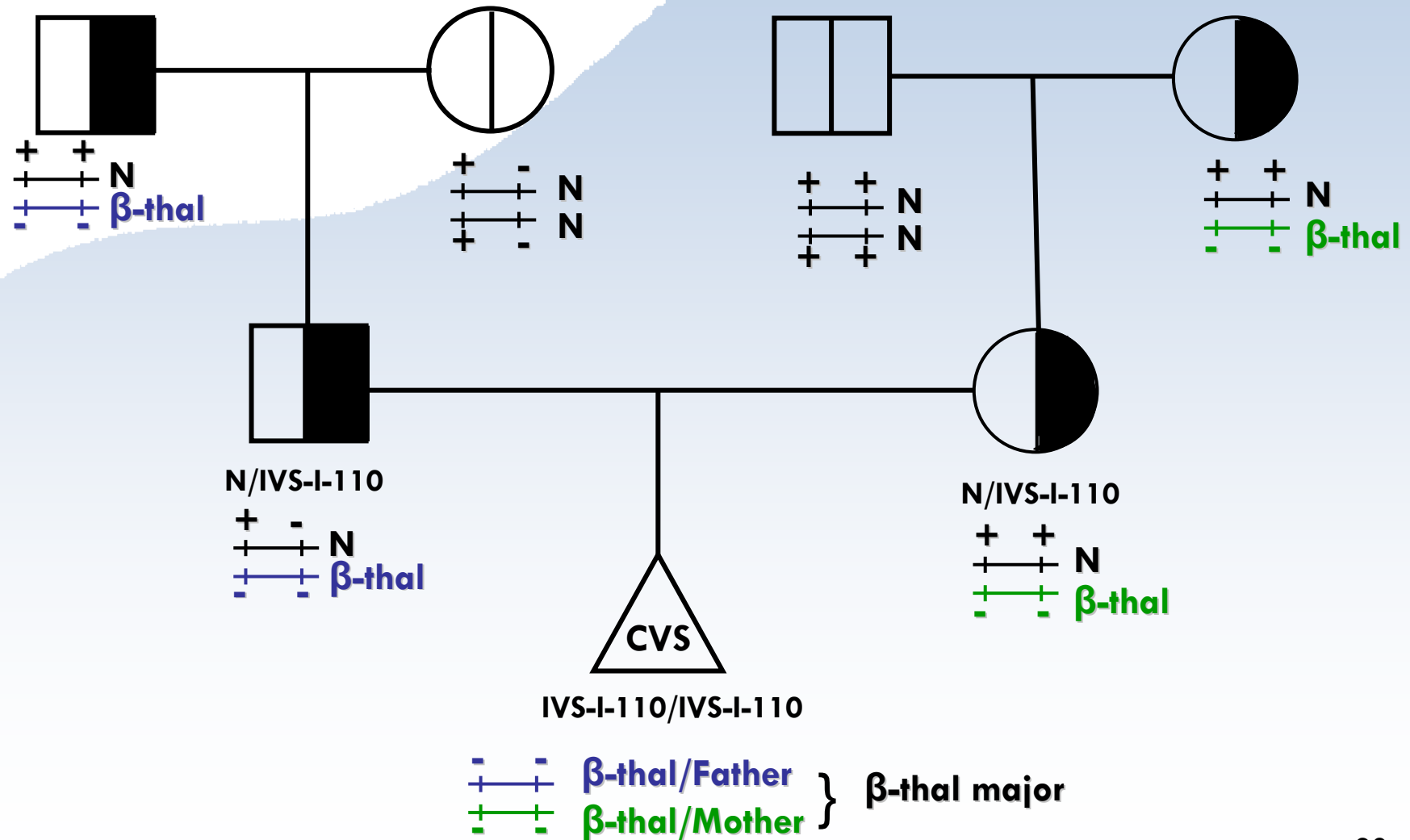
Typical couple at risk for β -thalassaemia



Typical couple at risk for β -thalassaemia



Typical couple at risk for β -thalassaemia



Atypical couple

One parent is a typical β -thalassaemia carrier while the other partner has abnormal haematological indices and normal HbA₂



α -thalassaemia
 δ and β thal comp. heter.
Silent β -thalassaemia
 α , β and δ thal comp. heter.
 β -thal with low HbA₂
 $\gamma\delta\beta$ -thal



Preimplantation Genetic Diagnosis



Preimplantation Genetic Diagnosis

- Preimplantation Genetic Diagnosis (PGD) uses in vitro fertilisation (IVF) to create embryos
- Tests one or two cells from each embryo for a specific genetic abnormality
- Identifies unaffected embryos for transfer to the uterus
- The approach through PGD assists couples at risk of an inherited disorder to avoid the birth of an affected child.



STAGES

- Counseling
- Induction of ovulation
- Oocyte collection
- Fertilization by ICSI
- Embryo biopsy
- Genetic diagnosis
- Implantation of 1-2 suitable embryos
- Confirmation of pregnancy
- Prenatal diagnosis (ESHRE guidelines)



Disorders tested by PGD

- FISH
 - Chromosomal Disorders
- PCR-based
 - Single gene defects
 - Thalassaemia
 - Cystic Fibrosis
 - Haemophilia
 - Muscular dystrophies
 - etc



PGD approaches

- Polar body analysis
- Blastomere biopsy analysis



Induction of ovulation

- In order to obtain a large number of oocytes, the patients undergo controlled ovarian stimulation (COH), with the use of FSH.
- Ultrasound-guided trans-vaginal oocyte retrieval

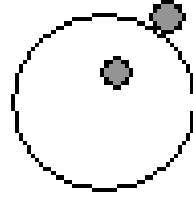
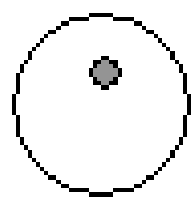


Fertilization

- Intracytoplasmic sperm injection (ICSI)
- Pronuclear formation (+ 2nd polar body)
- Pronuclear fusion
- Zygote



Immature oocyte



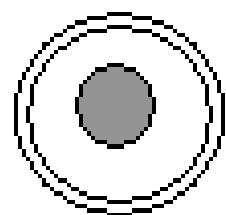
mature oocyte



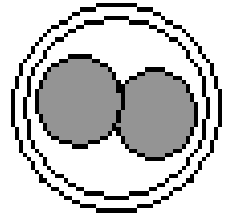
In vitro maturation



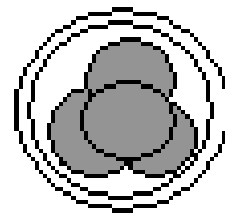
In vitro fertilisation



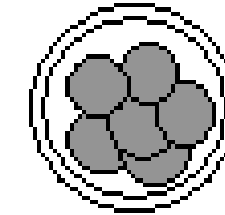
1-cell



2-cell



4-cell



8-cell

PGD

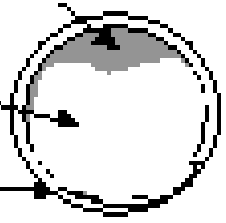


In vitro production

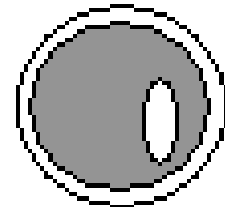
Inner cell mass

blastocoel

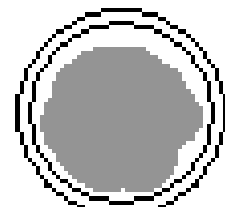
trophectoderm



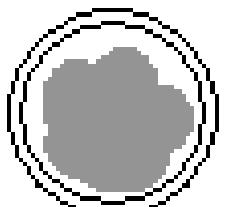
Expanded blastocyst



Early/Expanding blastocyst

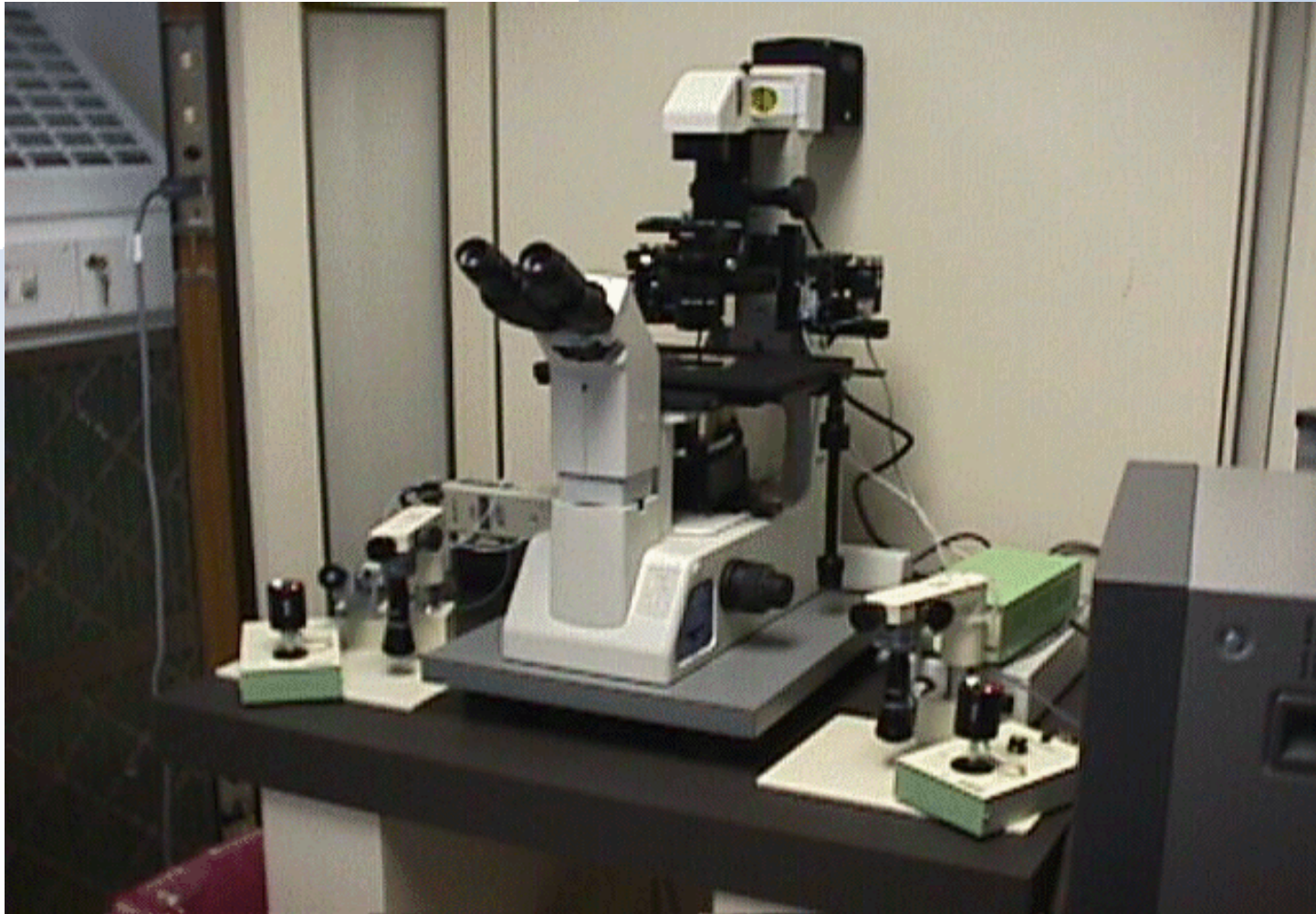


Morula



Compacting

Micromanipulator





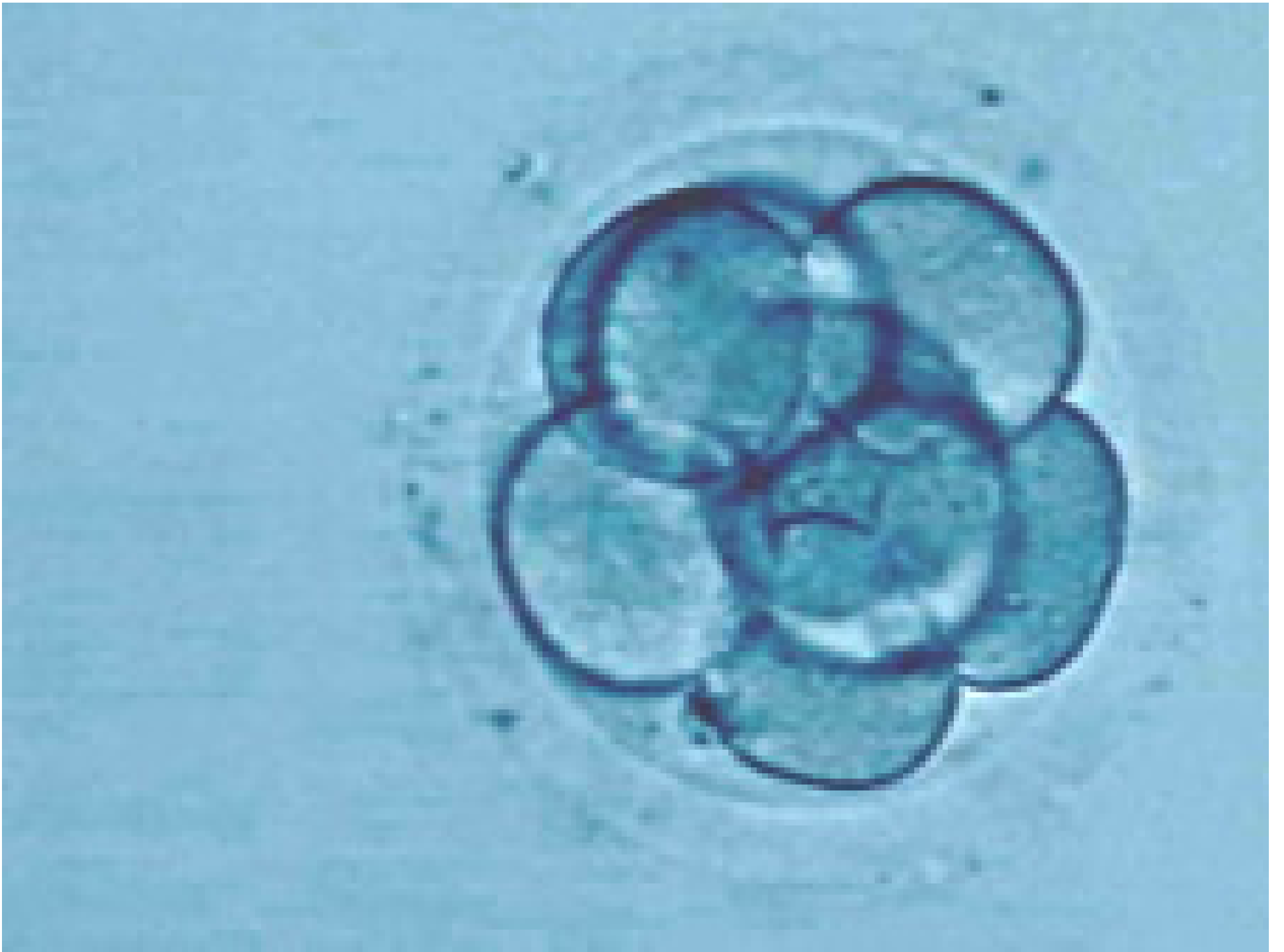


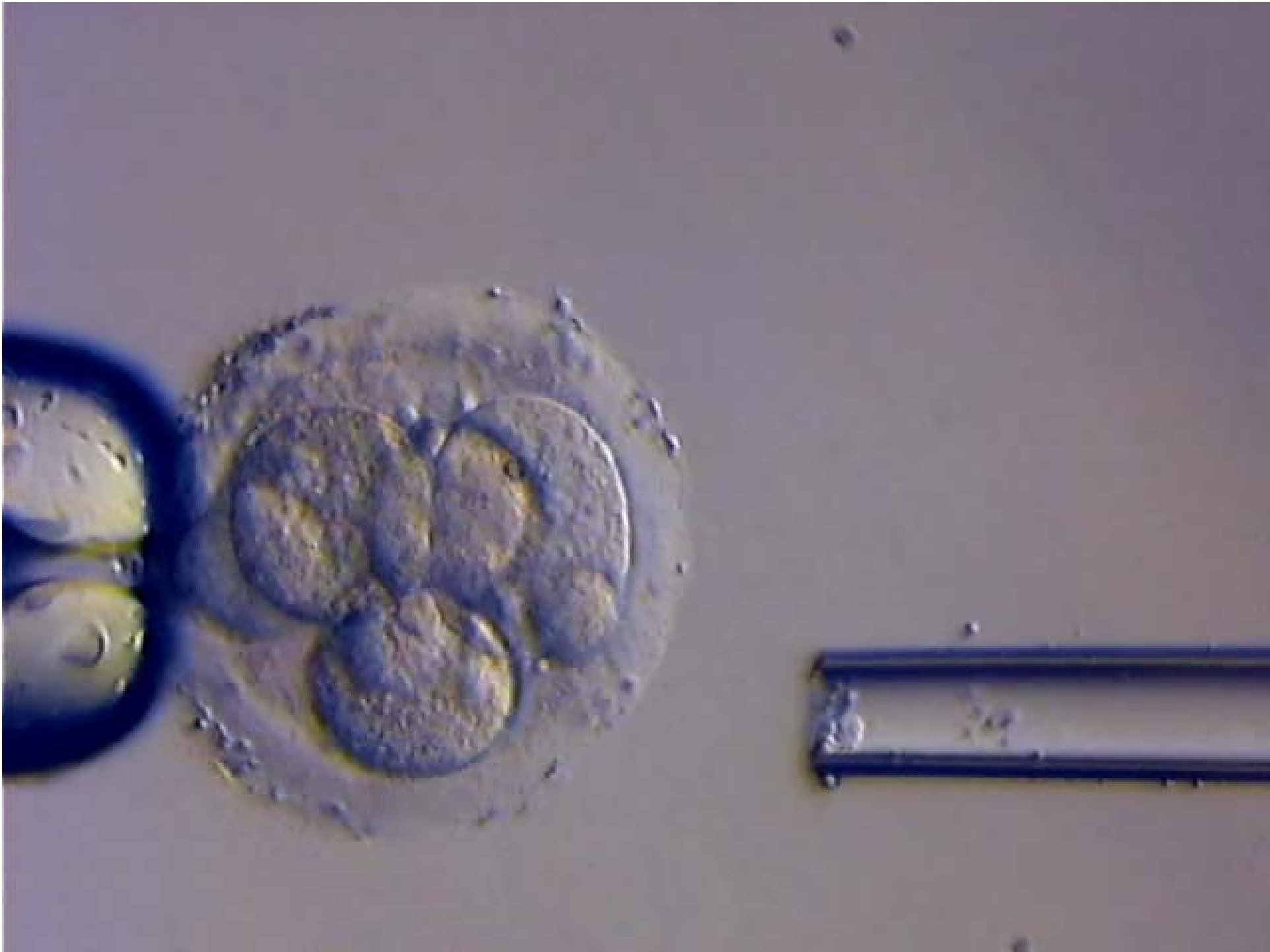
Second polar body extrusion and pronuclear formation following ICSI in a zona-free human oocyte



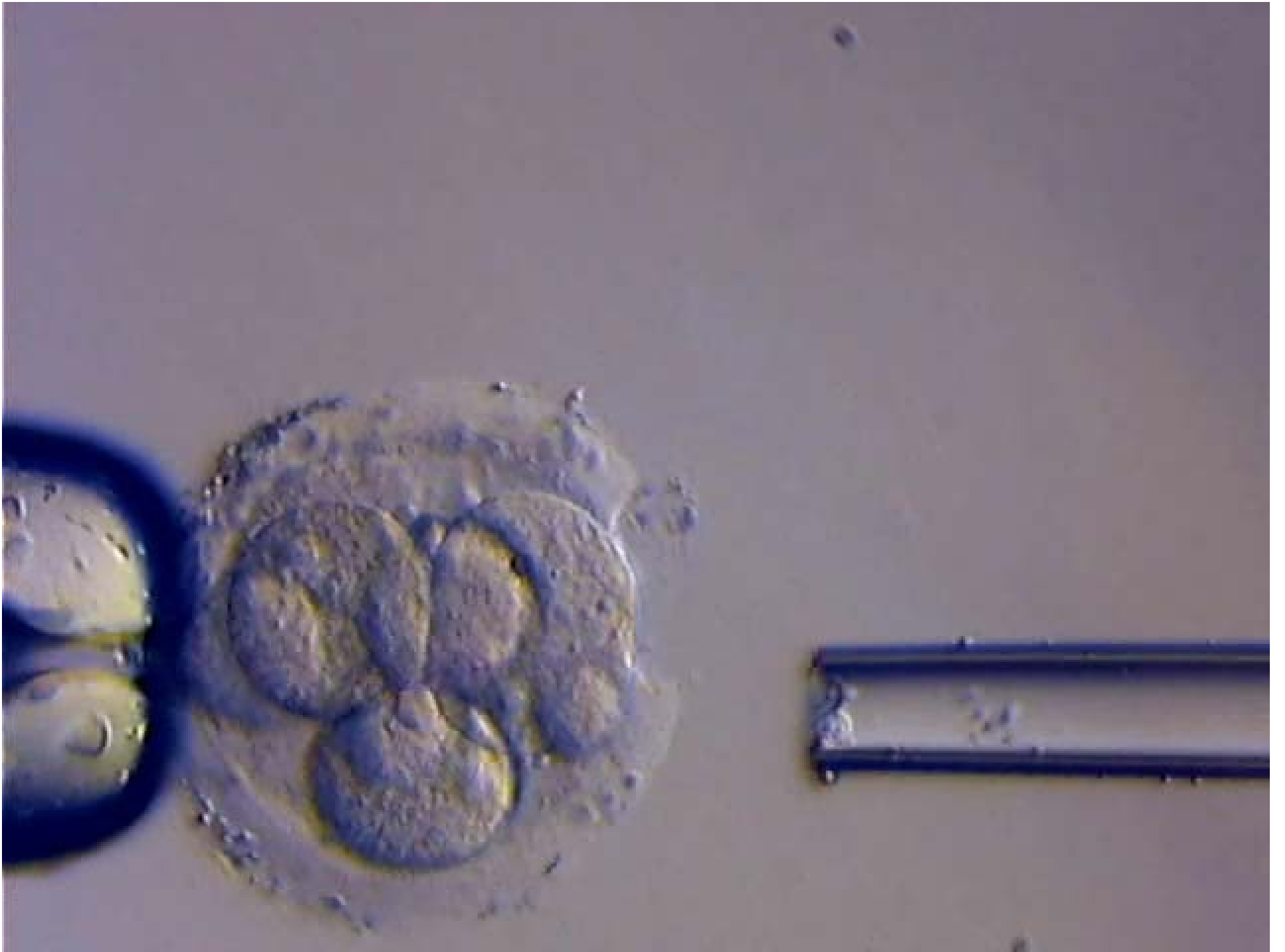












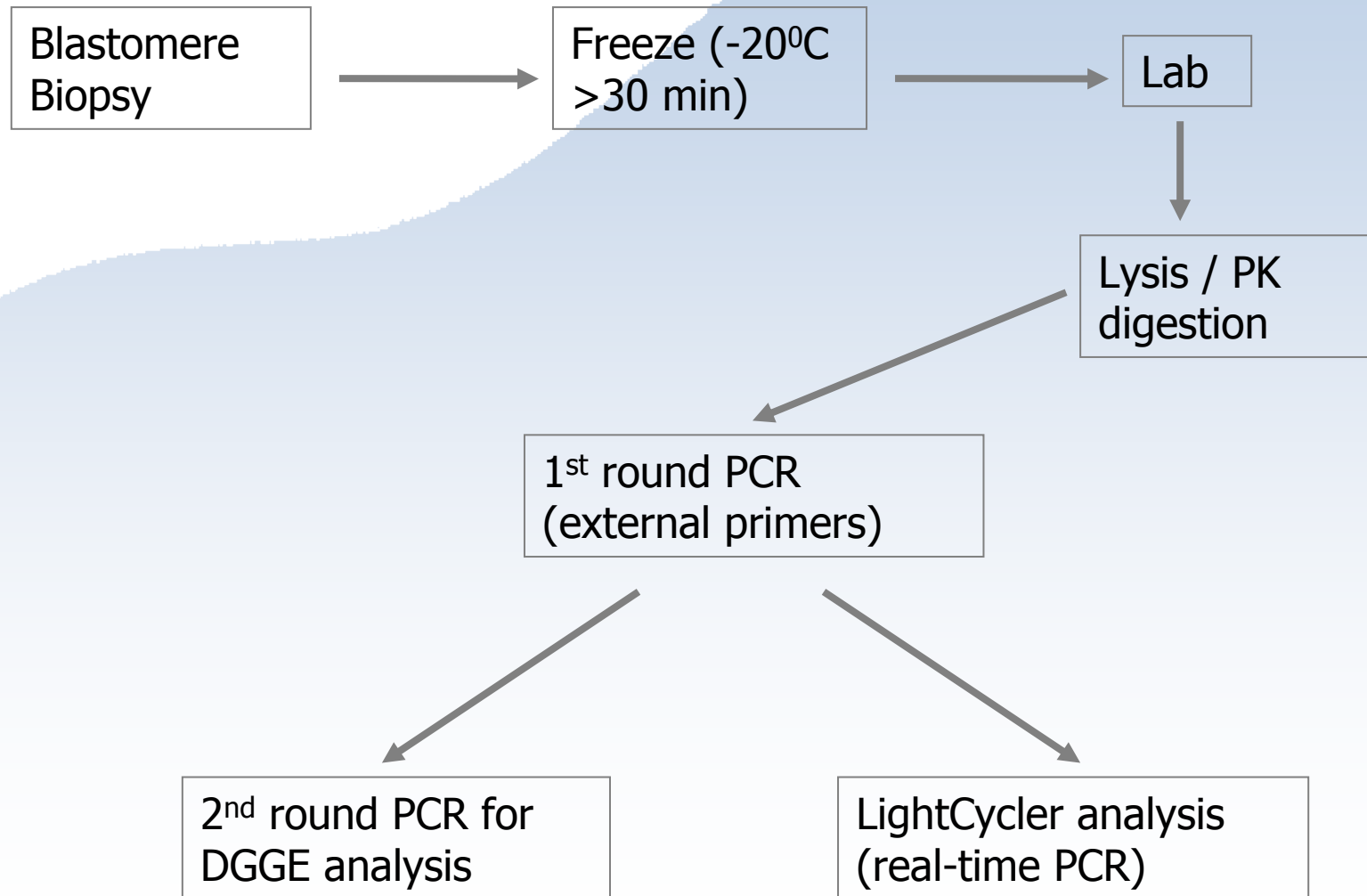




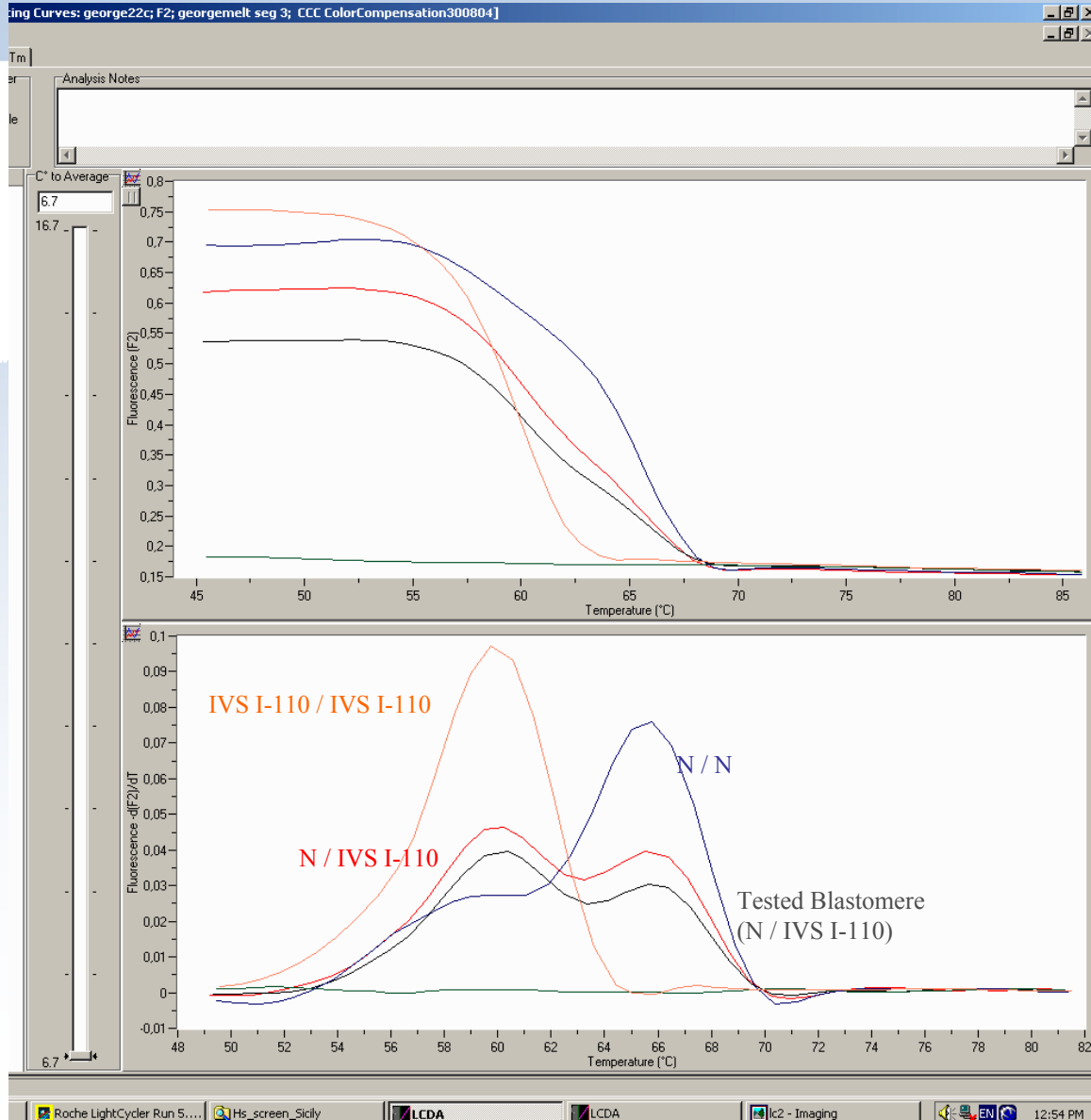


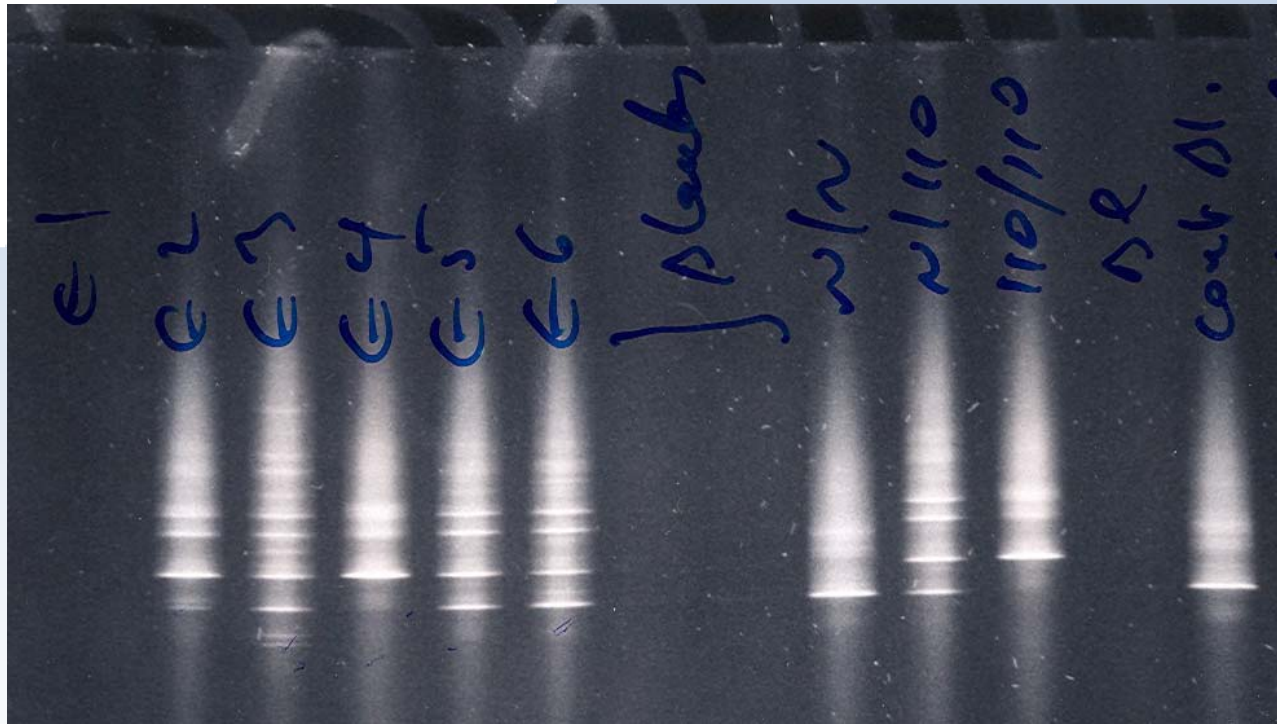


PCR-based PGD analysis



Melting curve analysis for the IVS I-110 mutation





DGGE analysis of 6 blastomeres during PGD



Preparation workup

- Strategy
- Training
- Setup of techniques on genomic DNA
- Tests on single cells (lymphocytes) >200
- Maximize amplification efficiency (>90%)
- Minimize allele dropout (<10%)
- Eliminate contamination factors
- Blastomere test from unused embryos



Determining factors for successful PGD

- Adequate number of ova
 - Not all will be fertilized successfully
- Adequate number of embryos
 - Not all will survive biopsy
 - Some may fail to develop normally
 - After analysis, ~25% expected not be suitable for transfer (affected)
 - A few may fail to amplify (5-10%) – no result
- Laboratory procedures
 - Biopsy techniques
 - Contamination control
 - Successful amplification of biopsy DNA



Sources of error

- Contamination
- Biopsy material (blastomere) actually not deposited in sample tube
- Cell fragmentation (bad quality embryos)
- Amplification efficiency



Contamination

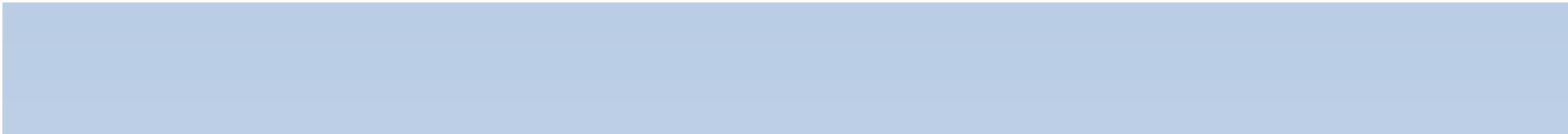
- Embryo manipulation
 - Handling
 - Biopsy
- Biopsy manipulation
 - Transfer
 - PK digest
 - First PCR amplification

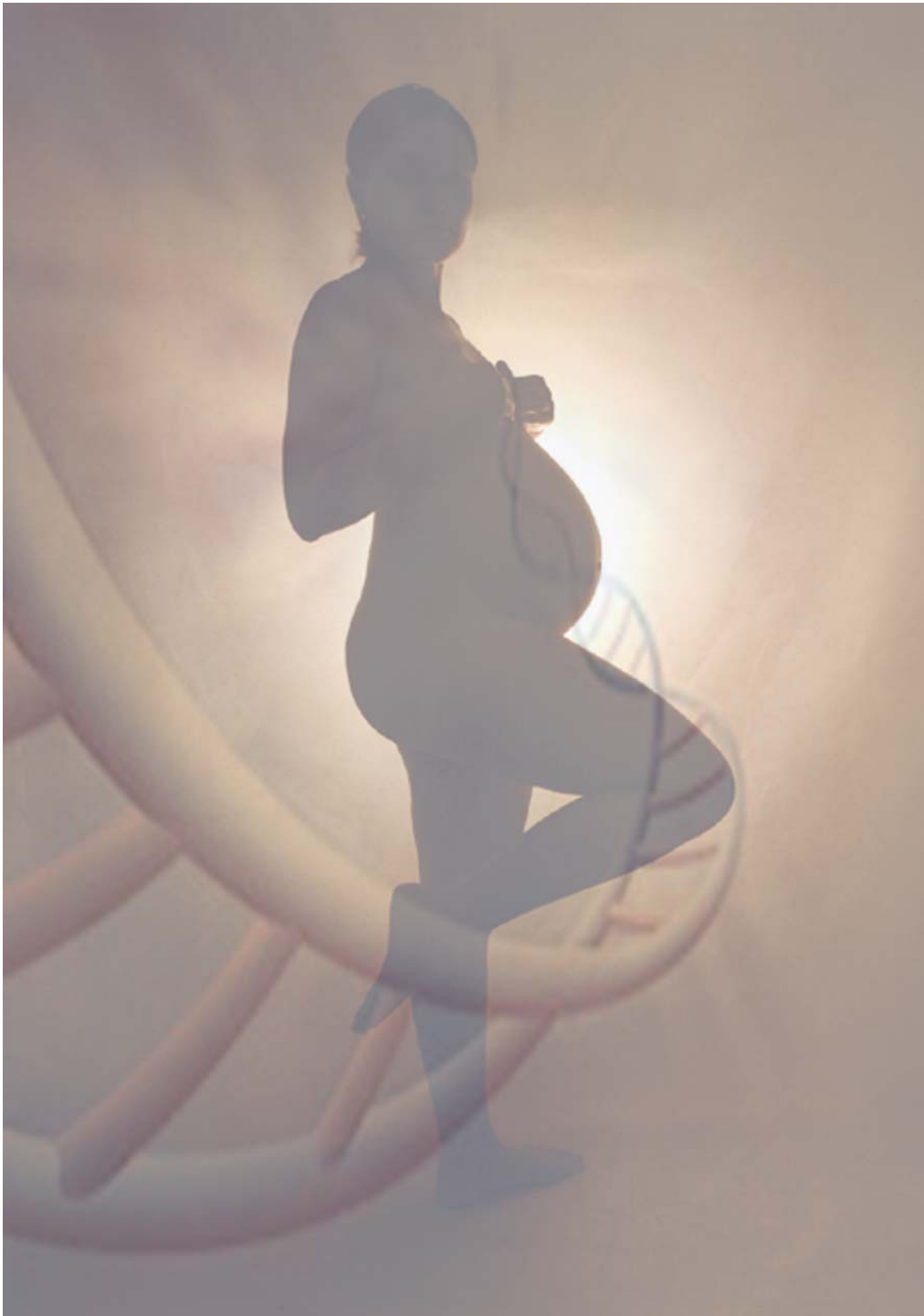


Contamination Control

- Blanks
 - Culture Medium Blanks
 - Biopsy Medium Blanks
 - Reagent Blanks
- Polymorphic Markers
 - D6S1056 (tetra-)
 - D15S652 (tri-)







Non Invasive Prenatal Diagnosis

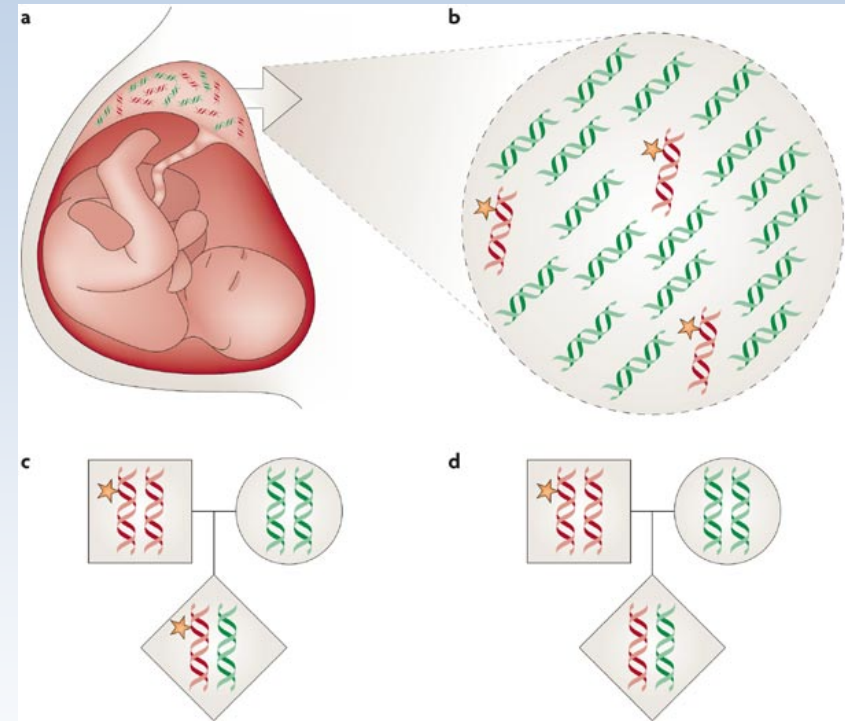
Fetal Cells in Maternal Circulation

- A very small amount of fetal cells are present in the maternal circulation
- Methods for separating FNRBCs failed to recover a pure fetal cell population
- New technologies are now tested
 - Non-contact laser capture microdissection
 - Separation by electric field



Circulating Nucleic Acids

- First report 1948 (Mandel and Metais)
- Studies on Circulatory DNA focused on autoimmune diseases
- Diagnosis and prognosis of cancer 1977
- Discovery of fetal DNA in maternal plasma (Lo et al, 1997)
- NIPD offered for RHD and fetal sex for X-linked disorders
- NIPD under development for other single gene and chromosomal disorders



Properties of fetal DNA

- Possible source (placenta)
- Increased in a variety of pregnancy-related pathologies
- Fragmented (< 300 bp)
- 3-6% of plasma DNA
- Differentially methylated



Development of NIPD methods Limitations

- Low quantity of fetal DNA
- Bad quality of fetal DNA
- The isolated DNA is mainly maternal (3-6% is fetal)
- Parents have the same mutations



NIPD Methodology

10ml peripheral blood



4 ml Plasma



Plasma DNA

(Maternal + Fetal)



PCR based methods



NIPD for X-linked Disorders

- Test for the presence Y chromosome sequences in the maternal plasma

Used for severe X-linked disorders:

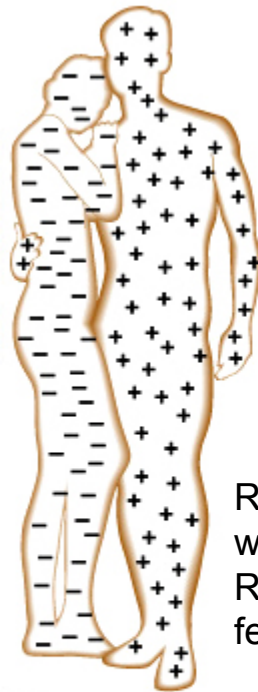
- Duchene/Becker muscular dystrophy
- X-linked agammaglobulinaemia
- Hemophilia
- Norrie disease (Episcopi blindness)
- X-linked severe combined immunodeficiency (SCID)



Hemolytic Disease of the Newborn (HDN)

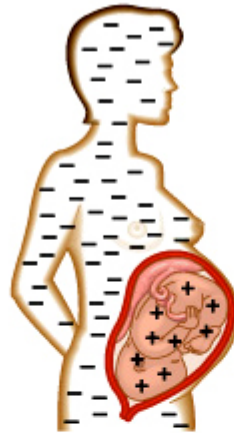
Mother: RHD-
Father: RHD+

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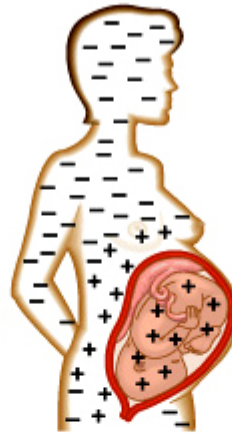


RhD-negative woman and RhD-positive man conceive a child

RhD-negative woman with Rh-positive fetus



Cells from RhD-positive fetus enter woman's bloodstream



Woman becomes sensitized
Antibodies form to fight RhD-positive blood cells



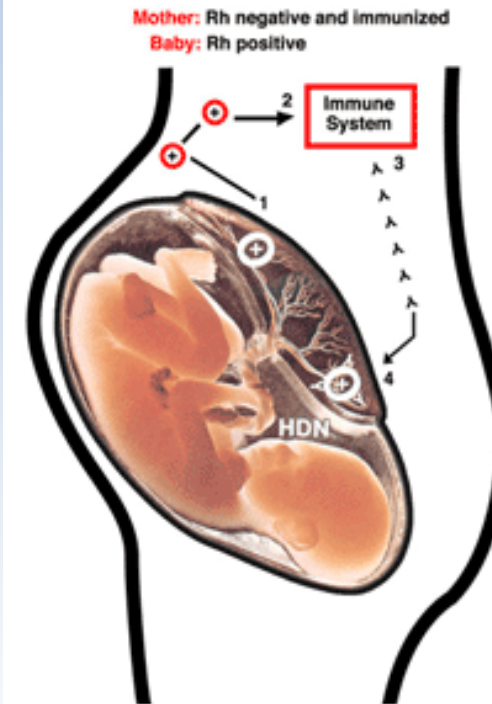
In the next pregnancy RhD-positive pregnancy, maternal antibodies attack fetal red blood cells



NIPD for the RhD of the fetus

- For RhD-negative women
- Blood sample from the mother after the 16 week of gestation
- Analysis of the plasma DNA
- Diagnosis

Figure 1: Pathogenesis of Rh Hemolytic Disease of the Newborn⁹



1. Fetal red cells enter maternal circulation at birth.

2. Red cells are recognized by the mother's immune system.

3. Mother is sensitized and produces antibody.

4. Antibody crosses the placenta and causes HDN.



β -Thalassaemia Non-Invasive Prenatal Diagnosis by Cell Free Fetal DNA

- The method is based on the detection of the paternally inherited fetal alleles



Selection/Analysis of SNPs for NIPD

- High degree of heterozygosity
- Informative SNPs
 - Mother **A/A**, Father **A/B** (determination of allele)
 - Mother **A/A**, Father **B/B** (confirmation of paternal allele)



Selection of SNPs with high degree of heterozygosity

- SNP genotyping analysis
- 130 SNPs located on the β -globin gene cluster (<http://www.ncbi.nlm.nih.gov>)
- 75 random samples (Cyprus Population)
- Sequenom[®] MALDI-TOFF Mass Array



Analysis on 67 families at risk for β -thalassaemia for 42 SNPs

Families	Informative SNPs
19	16-34
8	11-15
29	2-10
6	1
5	0



NIPD method using SNPs can be performed on **84%** of families



Thalassochip

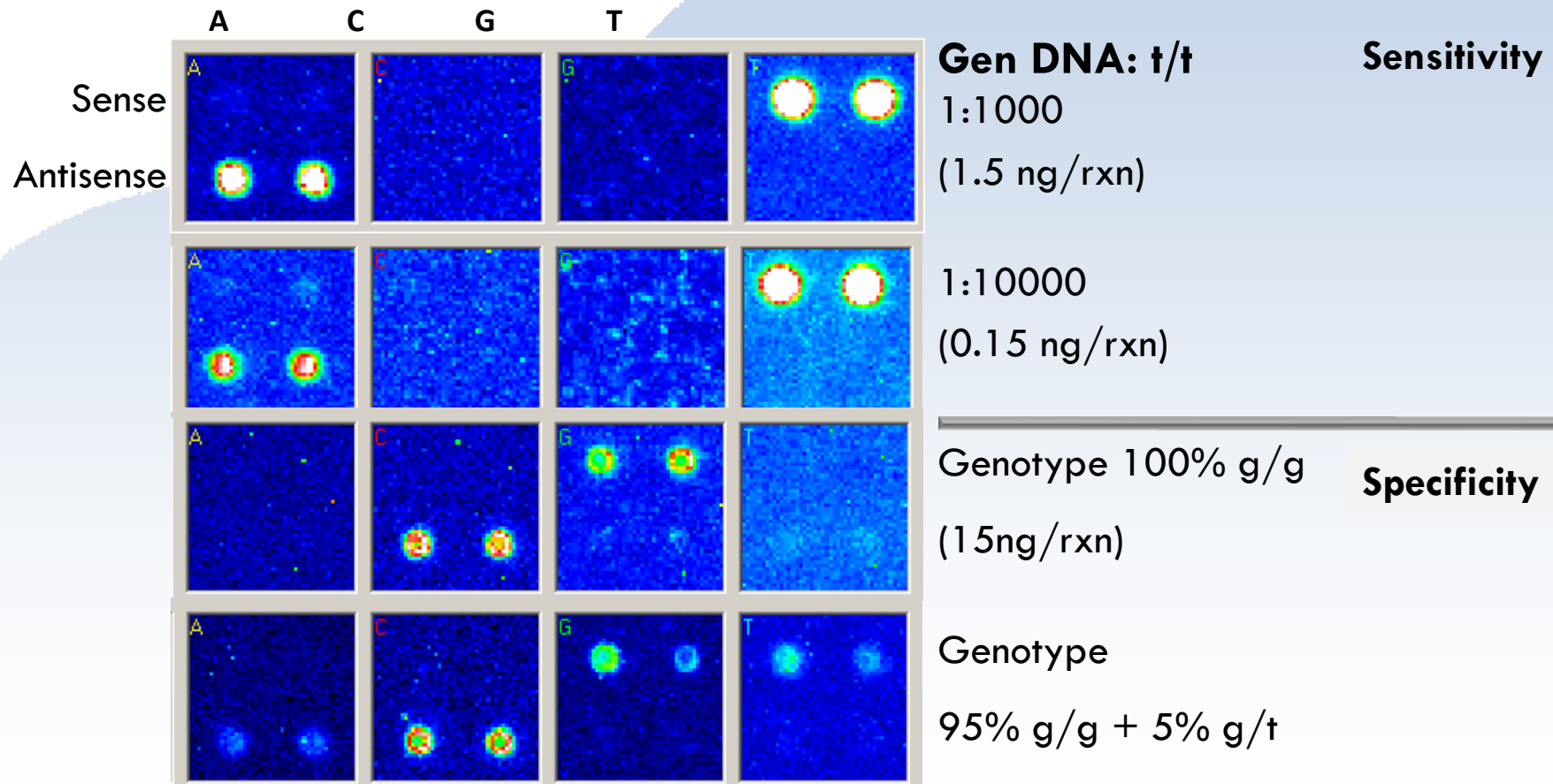
APEX (Arrayed Primer Extension)

- 54 beta thalassaemia mutations
 - 6 Hb Variants
 - 6 delta thalassaemia mutations
 - 10 SNPs
-
- It was validated as a diagnostic tool for haemoglobinopathies (EC MedGeNet project)

L. Cremonesi et al Hemoglobin 31:1-23, 2007



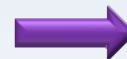
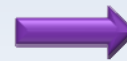
Sensitivity/Specificity of APEX gen DNA SNP rs7480526 (g/t)



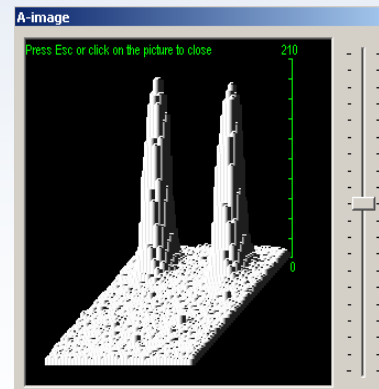
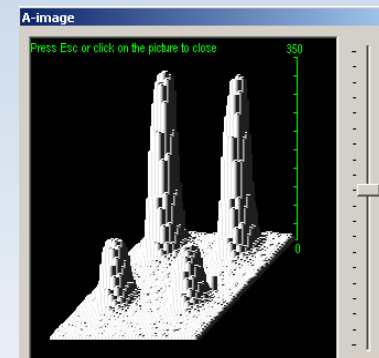
APEX analysis on maternal plasma

rs10837631(a/t) mo: a/a, fa:a/t

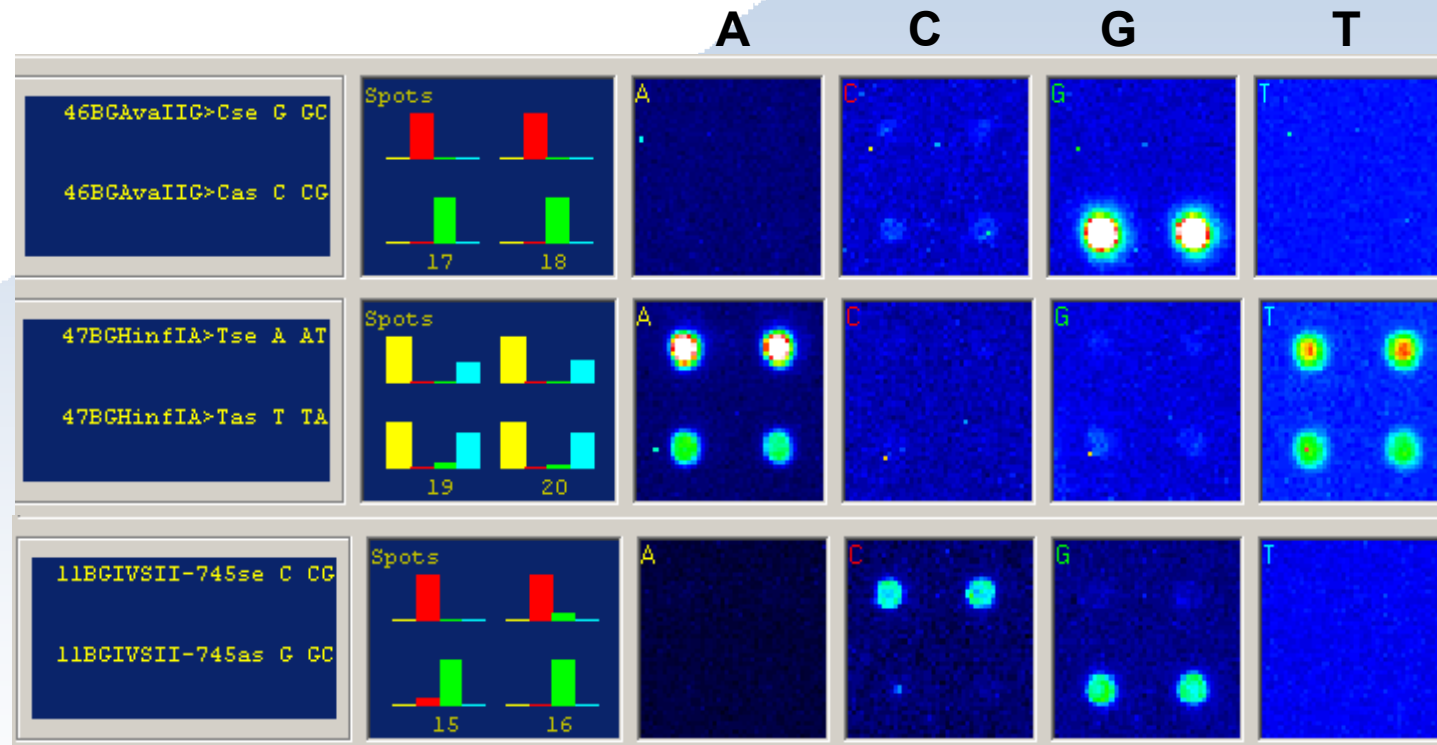
	A	C	G	T	CVS	Mat. Plasma
1						a/t a/t
2						a/a a/a
3						a/t a/t
4						a/t a/t
5						a/t a/t
6						a/a a/a
7						a/t a/a
8						a/t a/t
9						DNW
10						a/t a/t
11						a/t a/t



X



NIPD on Family 22



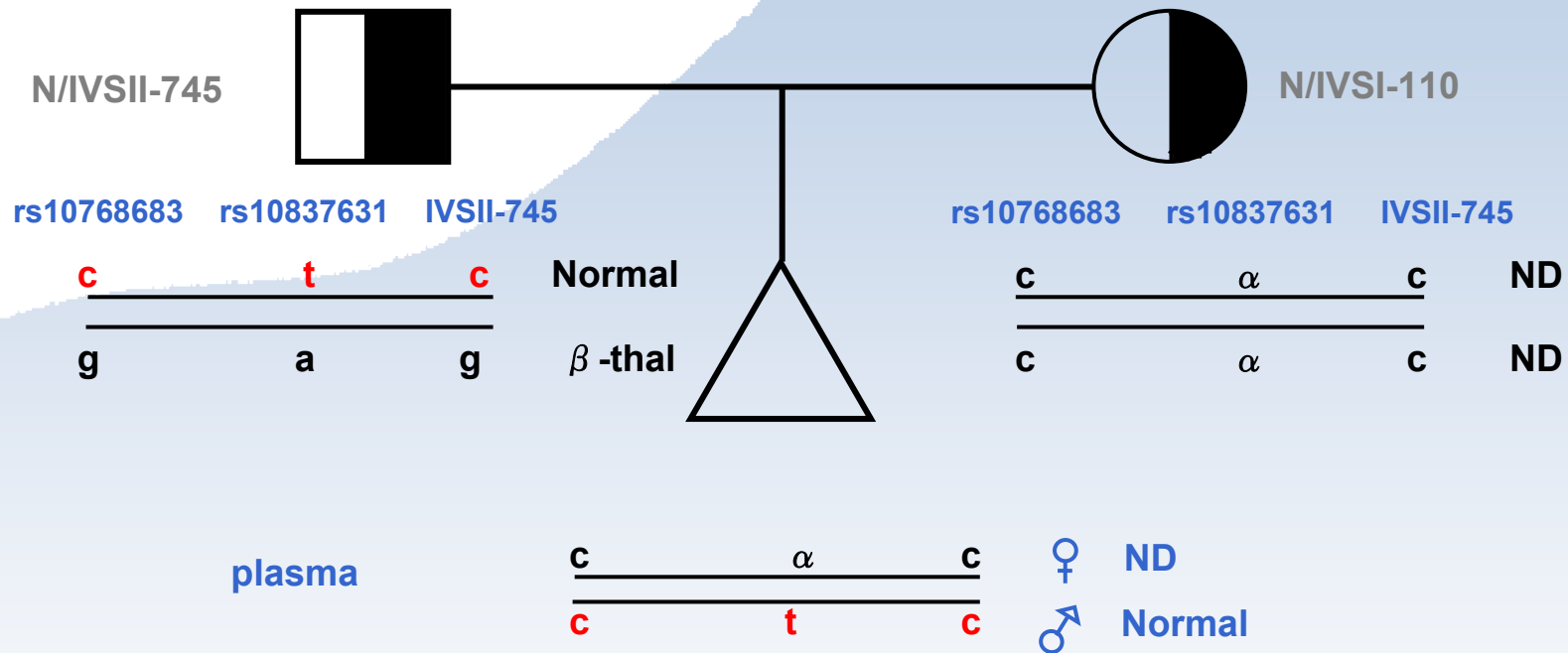
rs 10768683 c/g
 mother c/c
 fetus c/c

rs 10837631 a/t
 mother α / α
 fetus α / t

IVSII-745 c/g
 mother c/c
 fetus c/c



NIPD with SNPs (family 22)



NIPD: Normal or β -thal trait

Th. Papasavva et al, 2008. Ann. N.Y. Acad. Sci (In Press)



Conclusions

- NIPD using SNPs analysis is possible
- Risk of error reduces to acceptable levels using three or more SNPs (The higher the number of SNPs the more efficient/reliable NIPD.
- APEX-promising technique, needs improvement
- Paternal allele of the fetus non-invasively detected

