Genetic Testing Using Novel Technologies in ART

Basak Balaban, MSc
Alla Kalugina, MD, PhD
Filippo Maria Ubaldi, MD, MSc

Section 1
Genetic Testing and Male Infertility
Learning Objectives

After completing this section, participants should better understand:
- Etiologies and risks of genetic abnormalities of infertile men
- Genetic and molecular causes of male infertility
- Genetic screening of male infertility
- Role of genetic counselling
- Influence of genetic testing on ART outcomes

Genomics Era – Tools for Further Studies

- 10% of genes in the human genome may be related to spermatogenesis and fertility
- 400 candidate genes may be responsible for male infertility
- The genetics of male infertility in the era of genomics: a tool for progress

Prevalence and Risk of Genetic Abnormality of Infertile Men

- Risk exists for miscarriages and having children with chromosomal, congenital defects
  - Men with azoospermia: 10-15%
  - Men with severe oligozoospermia (<5 million/mL): 5%
  - Men with normal sperm concentration: 1%
  - Sex chromosomal aneuploidy (Klinefelter syndrome 47,XXY): 1.5-7%
  - Structural autosomal abnormalities (inversions, balanced translocations): 2%

Genetic Etiologies of Male Infertility

Sex determination/development
- Sex reversal
- Cryptorchidism
- Congenital bilateral absence of the vas deferens (CBAVD) and cystic fibrosis transmembrane conductance regulator (CFTR)
- Sickle cell anemia, β-thalassaemia
- Fanconi anemia
- Sperm production and function

Endocrinopathies
- Hypogonadism
- Pituitary/gonadotropin defects
- Steroid biosynthesis, metabolism and action
- Klinefelter syndrome
- Translocations, inversions, detections
- XX male, XY female
- Chromosomal (numerical/structural)
Abnormal karyotypes are less frequent if spermatogenesis is healthier

<table>
<thead>
<tr>
<th>Sperm concentration</th>
<th>Abnormal karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;20 million/mL</td>
<td>1.76%</td>
</tr>
<tr>
<td>&gt;20 million/mL</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>&gt;100 million/mL</td>
<td>0.2%</td>
</tr>
</tbody>
</table>

Intra-cytoplasmic sperm injection (ICSI) enables sperm resulting from severely defective spermatogenesis to bypass natural selection processes to initiate pregnancies.


Genetic or Molecular Causes of Male Infertility

- Numerical and structural chromosomal abnormalities
- Monogenetic disorders (cystic fibrosis, androgen receptor defects)
- Mitochondrial DNA mutations
- Multifactorial disorders (mutation in multiple genes often coupled with environmental factors)
- Infertile men with normal karyotype (including germinal mosaics)
Numerical and Structural Chromosomal Abnormalities

- 47,XXY
- 47,XYY
- Translocations – Robertsonian
- Translocations – reciprocal
- Inversions: paracentric and pericentric
- Y chromosome microdeletion


Klinefelter’s Syndrome (47,XXY)

- Frequency of sex chromosome aneuploidy varies from 1.5-7% in sperm from Klinefelter mosaics and 2-45% in non-mosaic 47,XXY karyotype
- Spermatogenesis seems to eliminate extra chromosomes
- Patients are prime candidates for ICSI

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### Outcome of ART in Klinefelter’s Syndrome

- Desirable to eliminate embryos with sex chromosome aneuploidy through preimplantation genetic diagnosis (PGD)
- Recovery of testicular sperm from men with non-mosaic Klinefelter’s syndrome (48%) was similar to other cases of non-azoospermia
- 36 healthy children were born after ICSI using sperm from non-mosaic Klinefelter’s patients with only one 47,XXY fetus identified


### 47,XYY

- Theoretically, 50% of sperm cells should be abnormal\(^1\)
- Case report: 75 sperm karyotypes from a 47,XYY male resulted in no disomic sperm for sex chromosome\(^1\)
- Increased incidence of sperm aneuploidy for sex chromosomes ranging from 0.3-15%
- Oligozoospermia may indicate more perturbations during meiotic pairing, subsequent loss of germ cells, and production of aneuploid sperm\(^2\)

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Translocations

- Robertsonian translocation carriers have a fusion of the long arms of 2 acrocentric chromosomes
- Risk of meiotic imbalance is primarily determined by characteristics of chromosomes involved and break-point positions
- Sperm karyotype studies of 37 reciprocal translocated heterozygotes have shown that 19-77% of spermatozoa are unbalanced
- Incidence of paternally derived translocation imbalances at prenatal diagnosis is about 12%; therefore, PGD is recommended


Inversions

- Inversion: 2 chromosome breaks occur in the same chromosome and then heal in an inverted order
- Correct number of genes, but an altered pairing of homologous chromosomes during meiosis
- If a single crossover occurs in the inverted region of the paired chromosomes, the presence of such recombinant chromosomes may result in an offspring with chromosomal duplications and deficiencies
  - Paracentric: same arm (risk of viable recombinants is ~3.8%)
  - Pericentric: both arms, including centromere (risk at prenatal diagnostic is 10-15%)


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**Azoospermia Factor (AZF)**

- AZF are located in three areas of Yq11: AZFa, AZFb, AZFc


**Y Chromosome Microdeletions**

- Incidence is 3-18% in men with severe sperm production abnormalities, including azoospermia
- Two-thirds of men with deletions in the azoospermia factor c region have sperm in ejaculate. Testicle production is present in azoospermic men. Recommended: testicular sperm extraction (TESE)
- Men with azoospermia factor b deletions are unlikely to have sperm in ejaculate or recover sperm with TESE. TESE is not recommended
- 9% of men with azoospermia factor a deletion have non-obstructive azoospermia and Sertoli cell-only pattern
  - Partial deletion: germ cells found on testis biopsy
  - Complete deletion: no sperm cells retrieved


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Monogenic Disorder and Multifactorial Disorders

- Monogenic genetic disorders occur as a direct consequence of a single gene being defective
  - Cystic fibrosis carriers (bilateral congenital absence of the vas deferens)
  - Defect in the androgen receptor
- Multifactorial disorders result from mutations in multiple genes, often coupled with environmental factors


Cystic Fibrosis Gene Mutations

- CFTR mutation rate
  - CBAVD: 50%
  - Obstructive azoospermia: 15%

Cystic Fibrosis Mutation Screening in CBAVD

- Strong association exists between male infertility caused by CBAVD and CFTR gene mutations
- Cases of obstructive azoospermia without CBAVD can be associated with CFTR gene mutations

### Results of the screening test for the CFTR mutation of the 5T allele

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Couples screened</th>
<th>One carrier n (%)</th>
<th>Two carriers n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IUI</td>
<td>552</td>
<td>23 (4.0)</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td>IVF</td>
<td>604</td>
<td>36 (5.9)</td>
<td>4 (0.7)</td>
</tr>
<tr>
<td>ICSI and MESA-TESE</td>
<td>1,350</td>
<td>98 (7.3)</td>
<td>9 (0.7)</td>
</tr>
<tr>
<td>Azoospermia(^a)</td>
<td>121</td>
<td>23 (19.0)</td>
<td>2 (1.7)</td>
</tr>
</tbody>
</table>

Note: \(^a\) Extrapolated from ICSI and MESA-TESE group
IUI: intrauterine insemination; IVF: in vitro fertilization; MESA: microsurgical epididymal sperm extraction


## Outcome of Chromosomal Abnormalities in Infertile Men

<table>
<thead>
<tr>
<th>Abnormality type per concentration category</th>
<th>Chromosomal abnormality per concentration category</th>
<th>Consequences for offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Azoospermia</strong> (gonosomal - 7, translocation - 1, translocation and inversion - 1)</td>
<td>15.2% (12/79)</td>
<td>NI-6 (M and CA)-2 M-1</td>
</tr>
<tr>
<td>0–1 million/mL (gonosomal – 3, translocation – 2, inversion – 4)</td>
<td>3.1% (10/319)</td>
<td>NI-8 (M and CA)-2</td>
</tr>
<tr>
<td>1–5 million/mL (gonosomal – 2, inversion – 1)</td>
<td>1.2% (3/251)</td>
<td>NI-2 M-1</td>
</tr>
<tr>
<td>5–10 million/mL (translocation–3)</td>
<td>1.4% (3/211)</td>
<td>(M and CA)-2 M-1</td>
</tr>
<tr>
<td>10–20 million/mL (gonosomal – 3, translocation – 3)</td>
<td>3.1% (6/191)</td>
<td>NI-3 (M and CA)-3</td>
</tr>
<tr>
<td>20 million/mL (translocation – 2, inversion – 2)</td>
<td>2.3% (4/172)</td>
<td>NI-2 (M and CA)-2</td>
</tr>
</tbody>
</table>

NI: Chromosomal abnormality without increased risk for miscarriage or child with congenital anomalies; M: Chromosomal abnormality with increased risk miscarriage only; M and CA: Chromosomal abnormality with increased risk miscarriage and child with congenital anomalies

**Future Novel Technologies**

- Microarrays: identification of gene expression profiles of infertile phenotypes
- Genomic analysis: determine differentially transcribed genes
- Proteomic: determine protein expression profiles of fertile and infertile men
- Metabolomic: mass spectroscopy and nuclear magnetic resonance spectroscopy can be used to create metabolite profiles
  - Clinical applications of metabolomics include gamete selection (assessing the best sperm to use for ART) and genomic testing (screening for aneuploidy)


**Epigenetics and Spermatogenesis**

- Several genes in testes are regulated through epigenetic mechanisms
- Hypermethylation (MTHFR, PAX8, NTF3, SFN, HRAS, JHM2DA, IGF2, H19, and others) is associated with poor semen parameters
- There is a direct correlation between epigenetic aberrations and spermatogenesis
- Environmental factors impact epigenome and male infertility
- ICSI and round spermatid injection (ROSNI) may increase the incidence of imprinting disorders and adversely affect embryonic development
The Role of Genetic Counseling

- Identifiable genetic risks
- Information and explanation of genetic defect causes
- Current PGD options to select embryos for transfer
- Avoid passing on genetic abnormality to children
- Transmitting genes causing male infertility is of less concern with the small risk of associated somatic disease, but potential parents need to be aware

Conclusions

- Men with non-obstructive azoospermia or severe oligozoospermia (<5 million/mL) are at increased risk for having a definable genetic abnormality
- Individuals with normal karyotypes may have abnormal cell lines in their testes
- It is necessary to offer karyotype and Y chromosome analysis before ICSI with such sperm
- Genetic counseling should be provided whenever a genetic abnormality is detected
- Genetic information helps improve ICSI outcomes
Section 2

Genetic Testing and Female Infertility Anomalies

Learning Objectives

After completing this section, participants should better understand:

- The etiologies and risks of genetic abnormalities of infertile women
- Genetic and molecular causes of female infertility
- Genetic screening for female infertility
- The role of genetic counseling
- Novel technologies for genetic testing of female infertility
Prevalence and Risk of Genetic Abnormality of Infertile Women

- In about 10% of female infertile subjects, genetic abnormalities could be present, including chromosome aberrations and single gene mutations.
- The frequency of chromosomal abnormalities in female infertility is about 5%:
  - 2.8% have numerical sex chromosome abnormalities
  - 2.2% have structural autosomal abnormalities


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Prevalence and Risk of Genetic Abnormality of Infertile Women

<table>
<thead>
<tr>
<th>Table 6</th>
<th>Classification of genetic causes of female infertility</th>
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<tbody>
<tr>
<td>Chromosomal abnormalities (homozygous or mosaic)</td>
<td></td>
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<tr>
<td>Sex chromosomes</td>
<td></td>
</tr>
<tr>
<td>Turner syndrome and gonadal dysgenesis with short stature</td>
<td></td>
</tr>
<tr>
<td>(45,X) mosaicisms such as 45,X/46,XX and 45,X/47,XXX</td>
<td></td>
</tr>
<tr>
<td>Xq isochromosomes: del(Xq); of(Xq); r(Xq); etc</td>
<td></td>
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<tr>
<td>Gonadal dysgenesis with Y-cell line</td>
<td></td>
</tr>
<tr>
<td>Mixed dysgenics (45,X/46,XY)</td>
<td></td>
</tr>
<tr>
<td>46,XY gonadal dysgenesis (Soyer syndrome)</td>
<td></td>
</tr>
<tr>
<td>True hermaphroditism with Y-cell line</td>
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<tr>
<td>Autosomal translocations</td>
<td></td>
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<tr>
<td>Robertsonian translocations</td>
<td></td>
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<tr>
<td>Reciprocal translocations</td>
<td></td>
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<tr>
<td>Inversions</td>
<td></td>
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<tr>
<td>Gene mutations</td>
<td></td>
</tr>
<tr>
<td>X-linked</td>
<td></td>
</tr>
<tr>
<td>Fragile X syndrome (FRAXA)</td>
<td></td>
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<tr>
<td>Kallmann syndrome</td>
<td></td>
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<tr>
<td>Complete androgen insensitivity syndrome</td>
<td></td>
</tr>
<tr>
<td>Autosomal</td>
<td></td>
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<tr>
<td>Complex genetic syndromes in which fertility is a minor manifestation</td>
<td></td>
</tr>
<tr>
<td>Infertility as major manifestation</td>
<td></td>
</tr>
<tr>
<td>Genes for binders of FSH and genes for LH and FSH receptors</td>
<td></td>
</tr>
<tr>
<td>Genes for GDF-11 receptor</td>
<td></td>
</tr>
<tr>
<td>BPH3 (bipolar, phallic, genital, epicanthic incursions)</td>
<td></td>
</tr>
<tr>
<td>Dandy-Walker syndrome</td>
<td></td>
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<tr>
<td>Foetal syndrome</td>
<td></td>
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<tr>
<td>Chromosomal aberrations confined to oocytes</td>
<td></td>
</tr>
</tbody>
</table>


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Numerical and Structural Chromosomal Abnormalities

- Structural aberrations
  - Translocations
  - Chromosomal inversions
  - Supernumerary and marker chromosomes
- Constitutional aneuploidies
  - Turner syndrome
  - 47,XXX
  - Down syndrome (trisomy 21)
- Aneuploidy in gametes
  - Maternal age effect

Structural Aberrations - Translocations

- Reciprocal or Robertsonian translocations
  - Reduction in fertility
  - Spontaneous abortions and birth defects
  - Meiotic process impediment\(^1\)
  - Production of genetically unbalanced gametes
  - Failure of meiosis and subsequent elimination of germ cells\(^2\)
  - If non-homologous pairing involves X and Y chromosomes during meiosis I, it will interfere with X inactivation, resulting in a lethal gene-dosage effect on the germ cells\(^1\)
  - Interactions of the translocation chromosomes with other parts of the nucleus may produce errors in meiosis and cell death\(^3\)

**Structural Aberrations – Inversions and Supernumerary and Marker Chromosomes**

- **Inversion** – formation of a pairing loop
  - Meiotic process impediment
  - Reduction in rate of recombination leads to a breakdown of meiosis
  - If crossing over occurs, unbalanced gametes can be produced

- **Supernumerary and marker chromosomes**
  - Carriers of marker chromosomes are at risk of infertility due to meiotic arrest and instability


**Constitutional Aneuploidies – Turner Syndrome**

- 45,X is the characteristic karyotype in Turner syndrome patients (1/5,000 – 1/10,000), occurring in ~55% of cases
- Frequency of all karyotypes associated with the syndrome
  
<table>
<thead>
<tr>
<th>Karyotype</th>
<th>Frequency of Turner Syndrome (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45,X</td>
<td>100</td>
</tr>
<tr>
<td>45,X/46,XX</td>
<td>5</td>
</tr>
<tr>
<td>45,X/46,XY</td>
<td>1</td>
</tr>
<tr>
<td>45,X/46,XXY</td>
<td>0.2</td>
</tr>
</tbody>
</table>

- Primary amenorrhea occurs in 25% and secondary amenorrhea in 0.5–1% of women with 45,X
- In 10% of patients with a 45,X cell line and up to 50% of women with 45,X/X chromosome mosaicism, pubertal development and menstruation can be present, but short-lived

Constitutional Aneuploidies – 47,XXX and Trisomy 21

- **47,XXX**
  - Incidence is 1/1000 females
  - The extra X chromosome is of maternal origin in 95% of cases and has a strong association with increased maternal age\(^1\)
  - Normal weight, height, and mental function are present
  - Normal pre-pubertal development and fertility are present, but with early onset of menopause (30 years of age)\(^2\)

- **Trisomy 21**
  - Frequency is 1/700 births
  - Rare possibility to reproduce

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Aneuploidy in Gametes – Maternal Age

- Risks of aneuploid gametes include trisomic offspring and pregnancy loss (25% in first trimester)
- Three hypotheses have been postulated:
  - *Production Line Hypothesis*: Oocytes that enter meiosis first are the first to be ovulated, and those entering last are ovulated last; the latter are more prone to non-disjunction\(^1\)
  - *Local Factors Hypothesis*: Ovarian environment compromised with aging, in terms of oxygen concentration, pH, and hormone concentration; implicated in progressive loss of normal chromosomal disjunction during later meiosis\(^2\)\(^-\)\(^4\)
  - *Limited Pool Hypothesis*: Oocytes depletion of the ovary leaves the remnants more prone to non-disjunction\(^5\)

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Monogenic and Multigenic Causes of Female Infertility

- Hypogonadotropic hypogonadism
  - Normosmic hypogonadotropic hypogonadism (nHH)
  - Kallmann syndrome (KS)
- Hypergonadotropic hypogonadism
  - Premature Ovarian Failure (POF)
  - Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED)
  - Blepharophimosis–ptosis–epicanthus syndrome (BPES) type 1
- Eugonadism
  - Spontaneous ovarian hyperstimulation syndrome (sOHSS)
  - Mullerian aplasia
  - Endometriosis
  - Polycystic ovary syndrome (PCOS)
  - Leiomyomata

Hypothalamic–Pituitary–Gonadal Axis

Gonadotropin releasing hormone (GnRH) is responsible for sexual development and reproductive function by acting on the hypothalamic–pituitary–gonadal axis.

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Hypothalamic–Pituitary–Gonadal Axis (con’t.)

- GnRH is released in a pulsatile fashion in order to bind to its cell surface receptor on pituitary gonadotropes. This binding induces follicle stimulating hormone (FSH) and luteinizing hormone (LH) synthesis.
- FSH and LH (gonadotropins) bind to their G-protein coupled receptors in the gonads. This binding induces steroids and gamete development.
- Sex steroids are responsible for the inhibitory negative feedback on the gonadotropin stimulus.
- GnIH (gonadotropin inhibitory hormone), inhibins, and antimullerian hormone (AMH) also play important roles in reproductive function.\(^1\,^2\)


Hypogonadotropic Hypogonadism

- Symptoms typical of estrogen deficiency include absence of breast development or hypoestrogenic amenorrhea due to absence of negative feedback to the hypothalamus and pituitary gland.
- GnRH deficiency.
- Sense of smell
  - If normal: normosmic hypogonadotropic hypogonadism (nHH)
  - If impaired: Kallmann syndrome (KS)
- A small percentage of patients can regain reproductive function with treatment.\(^1\)

Hypogonadotropic Hypogonadism - Etiology

- KAL1 gene mutations cause nHH/KS in 35-40% of patients\(^1\)-\(^2\)
  - Inheritance of KAL1 is X-linked recessive; only males are affected
- GNRHR gene mutations cause nHH in 4% of patients
  - First form of recessive autosomal inheritance of the pathology\(^3\)-\(^4\)
  - Variable phenotypes from complete absence of puberty to partial pubertal development or constitutional delay\(^5\)
  - GNRHR gene mutations do not solely cause KS; additional autosomal disease causative genes are involved\(^6\)
- CHD7 is the causative gene of CHARGE syndrome,\(^7\) but it can be mutated in nHH/KS patients without this syndrome
- nHH/KS phenotypic features are caused by 24 additional genes
  - Mainly ligand/receptor partners involved in GnRH regulation are impaired
- Mutations in 6 other genes determine combined pituitary hormone deficiency (CPHD)
  - Growth hormone deficiency associated with absence of 1+ pituitary hormones
  - Inheritance can be autosomal recessive or dominant, or X-linked recessive


Hypogonadotropic Hypogonadism – Etiology (con’t.)

Among the genes identified, only two (KAL1 and NR0B1) are X-linked recessive, while 12 are autosomal recessive and 6 are autosomal dominant

Hypogonadotropic Hypogonadism – Clinical Considerations

- Digenic/oligogenic gene mutation identification has complicated counseling of these patients
- A single mutated gene is sufficient to cause the pathology, and a second mutation can exacerbate the phenotype
- Mutation screening in FGFR1 (10%), CHD7 (6%), GNRHR (5%), and TACR3 (6%) is sufficient to cover 16% of KS and 25% of nHH patients, thus simplifying genetic counseling
- FGFR1 and CHD7 are inherited in an autosomal dominant fashion; thus screening for them could be sufficient to diagnose nHH/KS

Hypergonadotropic Hypogonadism - Phenotype

- Symptoms typical of estrogen deficiency: absence of breast development or hypoestrogenic amenorrhea due to lack of negative feedback to the hypothalamus and pituitary gland
- High levels of FSH and LH, indicating that the problem resides in the ovary
- Cardiac anomalies are found in one-half of patients and renal complications in one-third
- Dysgenetic gonads in patients phenotypically female but with a 46,XY karyotype (Swyer syndrome)
  - Swyer syndrome is caused by SRY mutations in 15% of cases

Hypergonadotropic Hypogonadism – Etiology

Hypergonadotropic Hypogonadism – Premature Ovarian Failure (POF)

- Patients (46,XX karyotype) show premature ovarian insufficiency
- 14 genes known to be causative, accounting for 15% of patients
- FMR1 gene is most commonly involved and most largely known, and causes Fragile X syndrome (FRAXA)
  - FRAXA is an X-linked dominant disorder; affected males show variable mental retardation, facial dysmorphism, and macroorchidism
  - FMR1 normally presents with 5-50 CGG trinucleotide repeats in an untranslated region; expansion of this trinucleotide from 50 to up to 200 repeats causes premutation. Premutated alleles in women predisposes to further expansion during meiosis
  - > 200 repeats in males are causative of FRAXA due to full inactivating mutation; the mechanism entails methylation of the FMR1 promoter
  - 16% of women carriers of the premutated allele will develop POF
- POF patients have a 3-4% risk of being carriers of the premutated allele if they are the only affected individual in the family. This incidence increases to 12-15% if another female is affected by POF in the pedigree

Hypergonadotropic Hypogonadism – Other Diseases

- Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED)
  - Systemic autoimmune disorder
- Blepharophimosis–ptosis–epicanthus syndrome (BPES), type 1
  - Rare autosomal dominant disorder caused by mutations in FOXL1 gene

Eugonadism - Spontaneous Ovarian Hyperstimulation Syndrome (sOHSS), Mullerian Aplasia, and Endometriosis

- Spontaneous ovarian hyperstimulation syndrome (sOHSS)
  - Caused by FSHR activating mutation\textsuperscript{1,2} in which receptor is constitutively active
  - Autosomal dominant inheritance
- Mullerian aplasia
  - Absence of the uterus and vagina
  - Affects 1/5000 women and 10% of women with primary amenorrhea\textsuperscript{3}
  - Unknown etiology
- Endometriosis
  - Inflammatory disorder resulting in pelvic pain and infertility
  - 5-10% of women between puberty and menopause affected; 7-fold increase if familial
  - Multifactorial and polygenic disease with unknown etiology; genome-wide association studies (GWAS) are ongoing

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Eugonadism - Polycystic Ovary Syndrome (PCOS) and Leiomyomata

- Polycystic ovary syndrome (PCOS)
  - Defined as hyperandrogenic anovulation with or without polycystic appearing ovaries
  - Hyperandrogenemia causes hirsutism
  - Higher levels of free estrogens result in increased risk of endometrial cancer
  - Hyperinsulinemia increases risk of type 2 diabetes
  - Most common cause of anovulation due to infrequent LH surges, affecting 5-8% of women
  - Unknown etiology; GWAS are ongoing

- Leiomyomata
  - Fibroids (benign smooth muscle tumors of the uterus) of clonal or somatic origin can cause bleeding/hysterectomy
  - More than 1/3 of women suffer from leiomyomata
  - Etiology still not well defined


Role of the Clinician in Counseling of These Patients

- Hypogonadotropic hypogonadism
  - FGFR1 and CHD7 should be tested for mutations by sequencing the DNA of all coding exons and splice junctions
  - TACR3 and GNRHR tests could also be included to diagnose up to 25% of nHH cases

- Hypergonadotropic hypogonadism
  - Karyotype to identify Turner syndrome
  - 46,XX patients with POF should be offered FMR1 testing by polymerase chain reaction (PCR) and Southern blot for triplet repeat expansion analysis

- GWAS are ongoing and will provide information about additional causative genes
Role of the Clinician in Counseling of These Patients (con’t.)

- Eugonadal disorders
  - sOHSS: FSHR gene DNA sequencing for protein-coding exons and splice junctions
  - Mullerian aplasia: WNT4 DNA sequencing
  - Endometriosis, fibroids, or PCOS: No reliable tests are currently available
- Structural or numerical chromosomal abnormalities
  - Preimplantation genetic screening (PGS) using comprehensive chromosome screening (CCS) analysis platforms should be proposed to women considering ART, especially women of advanced maternal age or translocation carriers

Future Novel Technologies

- Novel technologies to be used in the future include:
  - Targeted deep resequencing aimed at simultaneous screening of all genes involved in hypergonadotropic and hypogonadotropic hypogonadism
  - Whole exome sequencing
  - Whole genome sequencing
- The costs of these technique are progressively decreasing
- Logistical and ethical problems will emerge from the interpretation of the data these technologies provide, especially in terms of genetic counseling
Conclusions

- Genetic causes of female infertility vary from structural and numerical chromosomal imbalances to monogenic and multigenic conditions, mainly impairing the hypothalamic–pituitary–gonadal axis
- Comprehensive counseling exploiting currently available diagnostic tools is needed in order to inform the patient about prognostic perspectives
- PGD/PGS ensure encouraging outcomes especially when the cause of infertility is advanced maternal age
- New technology, such as molecular screening techniques, will bring new insight into the etiology of female infertility by increasing the throughput and decreasing the cost of analysis

Section 3
Genetic Testing: The Role of PGD/PGS in the Novel Technologies in ART
Learning Objectives

After completing this section, participants should better understand:

- Definitions of preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS)
- Indications for the use of PGD and PGS
- Technical aspects of PGD and PGS
- Advantages and disadvantages of different biopsy methods
- Controversies and perspectives in preimplantation genetic testing (PGT)

Definition of PGT

- **Preimplantation genetic testing** describes procedures involving the removal of one or more nuclei from oocytes (polar bodies) or embryos (blastomeres or trophoderm cells) to test for mutations in gene sequence or aneuploidy before transfer
- **Preimplantation genetic testing** includes PGD and PGS
Definition of PGD and PGS

- **Preimplantation genetic diagnosis (PGD)** is used when one or both parents carry a gene mutation or a chromosomal rearrangement and testing is performed to determine whether that specific mutation or an unbalanced chromosomal complement has been transmitted to the oocyte or embryo.

- **Preimplantation genetic screening (PGS)** is used when the parents are known or presumed to be chromosomally normal and their embryos are screened for aneuploidy.

Indications for PGD – High Risk

- Single gene disorders:
  - Carriers of autosomal dominant disorders (risk – 50%), such as myotonic dystrophy (DMPK).
  - Carriers of autosomal recessive disorders (risk – 25%), such as spinal muscular atrophy (SMA1).
  - Female carriers of X-linked disorders Duchenne/Becker muscular dystrophy (DMD).
  - Carriers of mutations such as BRCA-1 mutation.

- Known chromosomal abnormalities (deletions, translocations, inversions).

- When human leukocyte antigen (HLA) matching is performed.

Indications for PGS – Low Risk

- Advanced maternal age
- History of recurrent early pregnancy loss
- Repeated implantation failure
- Severe male factor infertility
- Sex selection or family balancing
- Other indications


Differences Between PGD/PGS

- Primary aim
  - PGD: identify embryos unaffected uniquely and specifically by an inheritable disease
  - PGS: identify euploid embryos for successful pregnancy
- Fertility
  - PGD: often fertile
  - PGS: infertile or subfertile
- Preliminary work-up
  - PGD: needed in order to determine needed assays
  - PGS: not needed
Methods of Genetic Analysis

- Fluorescence *in situ* hybridization analysis (FISH-analysis)
- Array comparative genomic hybridization
- Single nucleotide polymorphism (SNP) array
- PCR amplification and sequencing
- Real-time quantitative PCR
- Next-generation sequencing

Applications and Methods for PGT

9-chromosome FISH:
For known chromosomal abnormality, gender selection (X-linked mutation)

PCR and sequencing:
For autosomal single gene mutation, X-linked single gene mutation, HLA matching

24-chromosome screening platforms (aCGH, aSNP, qPCR):
Different applications according to the resolution of the platform. Mainly, chromosome copy number variations and chromosomes imbalances greater than 2 Mb are detectable
Fluorescence in Situ Hybridization (FISH)


PCR Amplification and Sequencing


Genetic Testing Using Novel Technologies in ART

Next Generation Sequencing

Evaluation of targeted next-generation sequencing–based preimplantation genetic diagnosis of monogenic disease

- 100% consistent with TaqMan Allelic Discrimination assay
- 100% consistent with reference lab genotypes
- 100% reliable
- Reasonable cost
- Turn-around time of 24 hours from biopsy to results
- Can detect chromosomal aneuploidies, translocations, and single-gene diseases using a single trophectoderm biopsy

Challenges of Single-cell PCR

- Quality of embryo
- Limited amount of DNA
  - Requires nested PCR or a high number of amplification cycles (>45)
  - Robust and high-fidelity polymerase
  - Hot-start PCR
- Allele dropout (ADO)
- Failed amplification
  - Use of linked polymorphic markers
- Meiotic recombination (cross-over)
  - Markers flanking the gene of interest
Biopsy Stage for PGD/PGS

Polar body biopsy¹,²
- Paternal and post-zygotic errors not detected
- Need 2nd PB biopsy
- High rate of false positives
- Impacts embryo development
- Expensive, time-consuming

Blastomere biopsy³,⁵
- Extensive experience worldwide
- Small reduction in embryo viability
- High impact of mosaicism
- Single cell analysis issue

Trophectoderm biopsy⁴,⁶,⁷
- More robust genetic analysis
- High clinical predictive value
- No impact of biopsy
- Low impact of mosaicism
- Reduced number of embryos/cycles
- Most cost effective


Polar Body Approach to PGD – Chromosomal and Single Gene Disorders (PB1 and PB2)
- Comparable prevalence of meiosis I and II errors
  - 1/3 are isolated events not detected by PB1 testing
- Limited diagnostic value of blastomere analysis
- >1/3 of meiotic errors are complex, indicating overall disturbance in female meiosis¹
- Accurate embryo genotype assessment requires combined oocyte and embryo testing, particularly for chromosomal disorders
- Detrimental impact of biopsy on embryo development²
- High false positive and false negative diagnosis rate³
- Expensive and time-consuming


Genetic Testing Using Novel Technologies in ART
**Blastomere Biopsy**

- **Advantages**
  - Diagnosis of hereditary parental abnormality
  - Possible sex determination
  - Sufficient time for diagnosis
  - Highest worldwide experience
- **Disadvantages**
  - Highest level of chromosome mosaicism at this stage
  - Limits in performing interphase FISH and molecular-genetic diagnosis (1 or 2 cells)
  - Single cell analysis

**Blastocyst Biopsy**

- **Advantages**
  - More DNA, so more robust diagnosis
  - Blastocysts have less mosaicism
  - Low error = low miscarriage rate (4%)
  - No damage to the embryos
  - Facilitates single embryo transfer
  - Least time-consuming and most cost-effective
- **Disadvantages**
  - aCGH and aSNP analysis turnaround times not compatible with fresh embryo transfer

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Genetic Testing Using Novel Technologies in ART
Methods of Blastocyst Biopsy

Trophectoderm biopsy without zona breaching at the cleavage stage


Methods of Blastocyst Biopsy (con’t.)

Trophectoderm biopsy with zona breaching at the cleavage stage

Conventional Parameters of Blastocyst Evaluation are not Predictive of Euploidy


Conventional Parameters of Blastocyst Evaluation are not Predictive of Euploidy (con’t.)


Genetic Testing Using Novel Technologies in ART
Prognosis Depending on Age and Cohort Size

<table>
<thead>
<tr>
<th># Day 5 embryos</th>
<th>% patients with normal embryos (% normal embryos)</th>
<th>&lt; 35 years old</th>
<th>35 – 39 years old</th>
<th>40 - 42 years old</th>
<th>&gt; 42 years old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg donors</td>
<td></td>
<td>99%</td>
<td>95%</td>
<td>79%</td>
<td>61%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>69%</td>
<td>68%</td>
<td>49%</td>
<td>34%</td>
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<tr>
<td>1-3</td>
<td></td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
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<tr>
<td></td>
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<td>73%</td>
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<td>100%</td>
<td>75%</td>
<td>95%</td>
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<td>97%</td>
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<td>7-10</td>
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<td>73%</td>
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<td>70%</td>
<td>51%</td>
<td>49%</td>
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<tr>
<td>&gt; 10</td>
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<td>80%</td>
<td>95%</td>
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<td>67%</td>
<td>70%</td>
<td>51%</td>
<td>67%</td>
</tr>
</tbody>
</table>

Calculated using 875 cycles, 4600 embryos
Euploidy decreased with age (P < .01) but NOT cohort size


Implantation After PGS

<table>
<thead>
<tr>
<th>RCT</th>
<th>Patient group</th>
<th>Fresh or freezing</th>
<th>Genetic method</th>
<th>IR after PGS for 24 chrom. vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yang et al. 2012</td>
<td>&lt;35</td>
<td>Day 5 biopsy, day 6 fresh transfer</td>
<td>aCGH</td>
<td>40% increase</td>
</tr>
<tr>
<td>Schoolcraft et al. 2011</td>
<td>&gt;35 (av. 39)</td>
<td>Day 5 biopsy, freezing, fresh transfer</td>
<td>aSNP</td>
<td>32% increase</td>
</tr>
<tr>
<td>Forman et al. 2013</td>
<td>&gt;35</td>
<td>Day 5 biopsy, day 6 fresh transfer</td>
<td>qPCR</td>
<td>32% increase</td>
</tr>
<tr>
<td>Scott et al. 2013</td>
<td>20-42 (av. 32)</td>
<td>Day 5 biopsy, day 6 fresh transfer</td>
<td>qPCR</td>
<td>28% increase</td>
</tr>
</tbody>
</table>

All randomised controlled trials (RCTs) show at least 30% increase in implantation rate (IR) after PGS for 24 chromosome analysis in comparison to no PGS


Genetic Testing Using Novel Technologies in ART
Clinical Evidence of Blastocyst Stage PGS: RCT

Table 3 Comparison of laboratory findings and clinical outcome among IVF patients undergoing SET with embryo assessment by aCGH + morphology (Group A) and blastocyst morphology alone (Group B)

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh blastocyst transfer according to morphology assessment</td>
<td>55 (100)</td>
<td>4 (100)</td>
<td></td>
</tr>
<tr>
<td>Grade 5/6</td>
<td>31 (56.4)</td>
<td>28 (58.3)</td>
<td></td>
</tr>
<tr>
<td>Grade 4</td>
<td>21 (38.2)</td>
<td>19 (39.6)</td>
<td>0.677^</td>
</tr>
<tr>
<td>Grade 3</td>
<td>3 (5.4)</td>
<td>1 (2.1)</td>
<td></td>
</tr>
<tr>
<td>Clinical pregnancy</td>
<td>39 (70.9)</td>
<td>22 (45.8)</td>
<td>0.017^</td>
</tr>
<tr>
<td>Ongoing pregnancy</td>
<td>38 (69.1)</td>
<td>20 (41.7)</td>
<td>0.009^</td>
</tr>
<tr>
<td>Miscarriage</td>
<td>1 (2.0)</td>
<td>2 (9.1)</td>
<td>0.592^</td>
</tr>
</tbody>
</table>

Notes: All data reported as n (%). SET = single embryo transfer; aCGH = array comparative genomic hybridization; GA = gestational age; ^ by Chi-squared test; by Fisher’s exact test.


Clinical Evidence of Blastocyst Stage PGS: RCT (con’t.)

• Females age <35 years
• aCGH
• Blastocyst stage biopsy on day 5 with fresh embryo transfer on day 6

Clinical Evidence of Blastocyst Stage PGS: RCT (con’t.)

The demographics of the comprehensive chromosomal screening (CCS) and control groups were equivalent as were basic parameters of follicular stimulation during their treatment cycles.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CCS study group</th>
<th>Nonintervention (control group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patrons (n)</td>
<td>72</td>
<td>83</td>
</tr>
<tr>
<td>Age (y)</td>
<td>32.2 ± 4.5</td>
<td>32.4 ± 0.5</td>
</tr>
<tr>
<td>Undergoing cycle donation (n)</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Mature follicles on day of hCG (n)</td>
<td>11.4 ± 1.1</td>
<td>12.4 ± 1.8</td>
</tr>
<tr>
<td>E2 (nmol/L)</td>
<td>2,412 ± 98</td>
<td>1,648 ± 92</td>
</tr>
<tr>
<td>Clinical diagnosis (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Preparation of embryos (%)</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

- Females age >35 years
- qPCR
- Blastocyst stage biopsy and day 6 fresh embryo transfer


66.4

47.9

Clinical Evidence of Blastocyst Stage PGS: RCT (con’t.)

- Female age 20-42 years (average 32)
- qPCR
- Blastocyst stage biopsy and day 6 fresh embryo transfer


Genetic Testing Using Novel Technologies in ART
Future PGD/PGS Strategy

- Improved diagnostic strategies for determining chromosome number
- Extend to microdeletions and microduplications
- Assessment may target genes essential for embryonic development
- Combination of single gene and aneuploidy screening
- Viability assessment (reduced time, accurate amplification, readily available, cost-effective)
- Combine chromosomal screening with novel genetic testing applications such as epigenetics and transcriptomics, from the same biopsy

Conclusions

- PGD is a complex and extensive process
- PGD is presently applied to a wide range of indications, including those of genetic or non-genetic nature, and is also combined with 24-chromosome aneuploidy testing
- Indications for PGD expand beyond diagnostic purposes and include treatment of siblings requiring HLA-compatible stem cell transplantation
Conclusions (con’t.)

- PGS offers
  - High-efficiency elective single embryo transfer
  - Increased pregnancy rate per cycle started
  - Faster time to pregnancy
  - Avoidance of unnecessary embryo transfers
  - Avoidance of cryopreservation of non-viable embryos
  - Prognostic information (recurrent IVF failure patients)

References

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- Plant TM. Hypothalamic control of the pituitary-gonadal axis in higher primates: key advances over the last two decades. *J Neuroendocrinol*. 2008;20:719-726.

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