Molecular diagnosis of Haemophilia

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Haemophilia

• is the **most frequent inherited coagulation disorders** due to the deficiency of FVIII (haemophilia A) and FIX (haemophilia B)

  - 1 in 5,000 is born with haemophilia type A
  - 1 in 30,000 is born with haemophilia type B

• the worldwide incidence of the disease is probably higher than this, as not all affected patients are diagnosed in developing countries
First case of haemophilia in the Talmud (male babies need not to be circumcized if two brothers had already died from the procedure)

Word “hemophilia“ has been used for the first time by Hopff at the University of Zurich

Haemophilia A associated with deficiency of a plasma component by Patek and Taylor (J Clin Invest, 1937)

Aggeler et al. (Proc Soc Exp Biol Med, 1952) and Biggs et al. (Brit Med J, 1952) recognized and described the two currently accepted types of haemophilia

Factor IX cDNA has been cloned (Kurachi & Davie. PNAS - 1982)

Factor VIII protein has been purified and its cDNA cloned (Gitschier J et al. Nature - 1984)

GENE THERAPY
Haemophilia A is also called the **Royal disease**, because Queen Victoria of England (1837–1901) was a carrier, and from her it spread to the royal families of Spain, Germany and Russia.
Haemophilia is an X-linked recessive blood coagulation disorder, therefore males usually are haemophilic patients and females are carriers.
Genetic analysis of severe haemophilia A patient

Direct analysis:

1. Intron 22 inversion (40-45%)
2. Intron 1 inversion (1-5%)
3. Sanger sequencing
4. MLPA
**F8 gene**

- is located towards the end of the long arm at Xq28
- spans **180 kb** and is one of the largest genes known
- comprises **26 exons** which encode a polypeptide chain of 2351 amino acids including a signal peptide of 19 amino acids
Six introns of the F8 gene are exceptionally large (>14 kb)

Intron 1 and intron 22 are particularly noteworthy because of their frequent involvement in pathological inversions, which are caused by recombination with homologous regions outside the F8 gene
• the most frequent inversion to affect F8 involves **intron 22** (32 kb) and is responsible for almost half of the cases of severe haemophilia A (40-45%)

• contains a CpG island, which acts as a bidirectional promoter for two genes **F8A (int22h-1)** and **F8B**
The mechanism of intron 22 inversion arises through **homologous recombination** between 9.5 kb region in intron 22 (int22h-1) and either of two extragenic distal homologous (int22h-2 and int22h-3) during meiosis.


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Long distance PCR protocol includes four primers:

P - Q are specific for 5’- and 3’-flanking region of int22h1 of F8C gene
A – B are specific for 5’- and 3’-flanking region of int22h2 and int22h3

(Liu et al, Blood 1998;92: 1458-1459)
### Long distance PCR (LD-PCR)

<table>
<thead>
<tr>
<th>Wild-type males or females</th>
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<tbody>
<tr>
<td>- PQ (12Kb)</td>
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<tr>
<td>- AB (10kb)</td>
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</table>

<table>
<thead>
<tr>
<th>Males with haemophilia</th>
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<tbody>
<tr>
<td>- PB + AQ (11kb)</td>
</tr>
<tr>
<td>- AB (10kb)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Females carriers</th>
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<tbody>
<tr>
<td>- PQ (12Kb),</td>
</tr>
<tr>
<td>- PB + AQ (11kb)</td>
</tr>
<tr>
<td>- AB (10kb)</td>
</tr>
</tbody>
</table>

In all cases, AB segment (10kb) serves as an internal control because at least one copy of int22h2 and int22h3 remains intact.

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Intron 1 inversion

Is an intrachromosomal recombination involving 1041bp specific duplicon between the two homologous sequences \textit{int1h-1} and \textit{int1h-2}. 

(Brinke A et al, 1996)
Detection of intron-1 inversion

The PCR assay for FVIII gene intron-1 inversion utilises:

- **three primers**: 9F, 9cr, int1h-2f specific for int1h-1 and for the sequence flanking int1h-2 in the centromeric side

- **three primers**: int1h-2f, int1h-2R, 9F specific for int1h-2 and for the flanking sequence at the telomeric side of int1h-1
Direct gene analysis in hemophilia A

- Detection of specific gene mutation is carried out by Sanger sequencing
- 5’ promoter sequence, all coding regions, intron/exon boundaries and the 3’ untranslated region (UTR) are amplified by PCR and sequenced
A large variety of mutations responsible for haemophilia A have been reported in HAMSTeRS Database (http://europium.csc.mrc.ac.uk)

- More than 1492 mutations within the $F8$ gene coding have been identified

- The mutations are heterogeneous and located throughout the $F8$ gene

- Point mutations are the most common type of mutation in the $F8$ gene
Multiplex Ligation-dependent Probe Amplification - MLPA

- First described in 2002 (Schouten JP et al. Nucleic Acids Res 2002: 30; e57)
- MLPA has a variety of applications including detection of duplications and deletions
- MRC-Holland offers MLPA probemixes for more than 250 different applications, e.g.
  - human genetics
  - cytogenetics
  - cancer research

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Multiplex Ligation-dependent Probe Amplification - MLPA

1. **Denaturation and hybridization**
   MLPA probe mix is added to denatured genomic DNA, and the two parts of each probe hybridise to adjacent target sequences.

2. **Ligation**
   Probes are ligated by thermostable ligase.

3. **PCR with universal primers**
   A universal primers pair is used to amplify all ligated probes.

4. **Fragment analysis**
   Separation and quantification by capillary electrophoresis.

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MLPA - Results

Wild type

Heterozygous Deletion

Homozygous Deletion

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Genetic analysis of severe haemophilia B patient

Direct analysis:
- Sanger sequencing
- MLPA

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Haemophilia B

• is often referred to as "Christmas disease" after the surname of the first child reported with this condition

• is an X-linked recessive bleeding disorder results from deficiency and/or defect in clotting factor IX

• The disease is caused by mutations in F9 gene, which is located in distal part of the long arm of the X chromosome (Xq27.1)
F9 gene

- has been completely sequenced in 1985 (Yoshitake S et al. Biochemistry 1985)
- spans 34 kb and comprises 8 exons
- mRNA is 2803 kb
- mature protein is 415 aminoacids

(Yoshitake S et al. Biochemistry 1985)
Direct gene analysis in hemophilia B

There is no counterpart of the intron 1 and 22 inversion for hemophilia B.

The strategy of mutation detection is based on direct sequencing or prescreening (DHPLC, High Resolution Melting) and then sequencing of abnormal fragments.

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Mutations in \( F9 \) gene

The mutations in \( F9 \) gene are regularly collected in a database freely accessible and mostly contain point mutations, small deletions and insertions.

There are currently 1113 unique variants in the F9 gene reported in Factor IX mutation database (http://www.factorix.org/).

<table>
<thead>
<tr>
<th>Unique Variants with specific mutation type</th>
<th>No. of mutations</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Point</td>
<td>812</td>
<td>72.9%</td>
</tr>
<tr>
<td>Deletion</td>
<td>182</td>
<td>16.3%</td>
</tr>
<tr>
<td>Insertion</td>
<td>39</td>
<td>3.5%</td>
</tr>
<tr>
<td>Indel</td>
<td>17</td>
<td>1.5%</td>
</tr>
<tr>
<td>Complex</td>
<td>5</td>
<td>0.4%</td>
</tr>
<tr>
<td>Duplication</td>
<td>4</td>
<td>0.3%</td>
</tr>
</tbody>
</table>

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Indirect molecular analysis in haemophilia
Indirect Molecular Analysis

The FVIII and FIX genes both contain two types of polymorphisms:
- **RFLPs** – restriction fragment length polymorphisms
- **VNTRs** - variable number of tandem repeats sequences

These markers are used to ‘track’ a defective (or normal) gene throughout an affected family in the context of hereditary disorders

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Limits of indirect diagnosis

- The mother of the index case should be heterozygous of the polymorphic marker

- When more than one polymorphic markers are utilised, a knowledge of linkage disequilibrium between these markers is absolutely essential

- The genetic distance between the polymorphic marker and the gene under investigation should be know because the risk of recombination is higher

- The informativeness of the polymorphic markers depends on the prevalence of the polymorphic sites in a given population
Prenatal Diagnosis in Haemophilia
Preimplantation genetic diagnosis

Non-invasive methods:
Fetal sex determination on maternal plasma or serum

Mutations research in hemophilic patient

Female carrier

Prenatal diagnosis:
- Chorionic villus sampling
- Amniocentesis

Preimplantation genetic diagnosis
Prenatal diagnosis in haemophilia

- To detect a genetic defect present in the embryo previously found in a carrier of haemophilia
- Molecular analysis applied on DNA sample extracted usually from chorionic villus in order to distinguish an affected fetus from an unaffected (sample could be also obtained from amniotic fluid)
Chorionic villus sampling

- it is usually performed in 10-12\textsuperscript{th} gestational weeks
- the sample should not get contaminated with any maternal DNA
- chorionic villus samples obtained before 10\textsuperscript{th} week of gestation is associated with a high risk of fetal limb abnormalities
- \textbf{fetal loss} attributable to a standard procedure is \textbf{approximately} 1%

All techniques used to obtain samples are \textbf{invasive} and never free from \textbf{risk} to the gravida or fetus and should be applied only when strictly required (religion, custom and all aspects have to be evaluated)
Non-invasive prenatal diagnosis

- Free fetal **DNA** in maternal blood
- Fetal **cells** in maternal blood
The discovery of free fetal DNA in maternal plasma is revolutionizing the field of prenatal diagnosis (Lo et al., 1997)

Free fetal DNA

- originates from placental cells, trophoblast (Alberry et al., 2007)
- can be detected from four weeks’ gestation (Illanes et al., 2007)
- is cleared from the maternal circulation, with an half-life of 16 minutes, and it is undetectable two hours after delivery (Finning & Chitty, 2008)
offered an alternative source of fetal genetic material for prenatal diagnosis allowing the development of ‘non-invasive prenatal diagnostic tests’ without risk to the pregnancy
Fetal sex determination is possible by:

- sample of peripheral maternal blood
- **extraction of fetal DNA** from maternal plasma sample
- amplification of specific DNA sequences on chromosome Y by **quantitative real-time polymerase chain reaction**

This procedure could

- reduce the procedure-related **risks** of an invasive technique
- reduce **psychological stress and anxiety** of pregnant women
Our experience in Milan:

- a cohort of **156 pregnant women** sampled throughout pregnancy, 74 delivered male and 82 female babies

- **low sensitivity at 4 to 7 weeks**, 73%.
- **after 8th** weeks of gestation the sensitivity is higher, 94%

<table>
<thead>
<tr>
<th>Table 1. Statistical parameters of the study.</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td>Sensitivity</td>
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<tr>
<td>Specificity</td>
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<tr>
<td>Accuracy</td>
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<tr>
<td>Positive predicted value</td>
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<tr>
<td>Negative predicted value</td>
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</tbody>
</table>

**Sensitivity** - number of male-bearing pregnancies identified/total number of male-bearing pregnancy tested;
**Specificity** - number of female-bearing pregnancies identified/total number of female-bearing pregnancy tested;
**Accuracy** - total number of male - and female-bearing correctly identified/total number of pregnancy tested

(Mortarino et al. Haemophilia 2011;17:952-956)
Noninvasive Fetal Sex Determination Using Cell-Free Fetal DNA
A Systematic Review and Meta-analysis

Stephanie A. Devaney, PhD
Glenn E. Palomaki, PhD
Joan A. Scott, MS, CGC
Diana W. Bianchi, MD

- Systematic review and meta-analysis with search of PubMed (January 1, 1997-April 17, 2011) to identify English-language human studies reporting primary data

- From 57 selected studies, 80 data sets (representing 3524 male-bearing pregnancies and 3017 female-bearing pregnancies) were analyzed
Gestational age had the largest effect on test performance

- prior to 7 weeks → sensitivity, 74.5% and specificity, 99.1%
- 7 through 12 weeks → sensitivity 94.8% and specificity, 98.9%
- 13 through 20 weeks → sensitivity 95.5% and specificity, 99.1%

(Devaney et al. JAMA 2011)

### Table 1. Test Performance

<table>
<thead>
<tr>
<th>Variable</th>
<th>Data Sets, No.</th>
<th>PPV</th>
<th>NPV</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Diagnostic OR</th>
<th>%</th>
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<td>96.7</td>
<td>97.2 (96.5-97.9)</td>
<td>98.7 (98.1-99.2)</td>
<td>903 (527-1548)</td>
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<td>93.5</td>
<td>93.9 (92.6-95.1)</td>
<td>99.2 (98.5-99.6)</td>
<td>863 (555-2100)</td>
<td>56</td>
<td>97.3</td>
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<td>31-46</td>
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<td>93.7 (93.5-96.6)</td>
<td>657 (86-5019)</td>
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<td>≤2003</td>
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<td>Plasma</td>
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<td>Urine</td>
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<td>Test method</td>
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<td>Gene</td>
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<td>Gestational age, mo</td>
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<tr>
<td>&lt;7</td>
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<td>98.8</td>
<td>80.6</td>
<td>74.5 (65.1-82.5)</td>
<td>99.1 (95.2-99.9)</td>
<td>116 (31-437)</td>
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<td>95.7</td>
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<tr>
<td>7-12</td>
<td>21</td>
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<td>95.1</td>
<td>94.8 (93.1-96.2)</td>
<td>98.9 (98.0-99.5)</td>
<td>846 (572-1925)</td>
<td>28</td>
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<tr>
<td>13-20</td>
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<td>93.3</td>
<td>95.5 (93.5-97.1)</td>
<td>99.1 (97.5-99.3)</td>
<td>604 (271-1347)</td>
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<tr>
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<td>99.0 (97.7-99.6)</td>
<td>99.6 (98.6-99.9)</td>
<td>3196 (710-14397)</td>
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<td>95.4 (94.7-96.1)</td>
<td>98.6 (98.1-99.0)</td>
<td>885 (565-1386)</td>
<td>32</td>
<td>96.8</td>
</tr>
</tbody>
</table>

(Devaney et al. JAMA 2011)
• noninvasive test using microfluidics digital PCR to diagnose a causative mutation for hemophilia in maternal plasma DNA

• fetal genotypes were detected in 12 maternal plasma samples from as early as the 11th week of gestation.
Noninvasive prenatal diagnosis empowered by high-throughput sequencing

Rosso W. K. Chiu* and Y. M. Dennis Lo

High-throughput sequencing has been shown to be an effective tool for the detection of aneuploidies.

A number of studies showed that massively parallel maternal plasma DNA sequencing can offer highly accurate detection of fetal Down syndrome.

One day it may be possible to diagnose monogenic diseases by massively parallel sequencing of maternal plasma DNA.
Fetal cells in maternal blood

- In 1893, a German pathologist, first documented the presence of fetal-derived trophoblast in the lung tissue of 17 pregnant women died from eclampsia.

- Definitive incidence came in 1969 when lymphocytes carrying an X and a Y chromosome were detected in the peripheral blood of pregnant women carrying male fetuses (Walknowska et al, Lancet 1969;1:119-1122).
Fetal cells in maternal blood

- are rare in maternal blood, range from 1 in $10^5$ to 1 in $10^9$ cells in maternal blood
- are a variety of fetal cell types present in maternal blood, trophoblasts, lymphocytes, erythroblasts or fetal nucleated red blood cells (FNRBCs) and hematopoietic stem cells (HMCs)

Methods of cell separation are:
- fluorescence activated cell sorting (FACS)
- magnetic activated cell sorting (MACS)
Isolation of fetal cells from maternal circulation

Magnetic activated cell sorting (MACS)

1. The cells of interest are labeled with MACS microbeads (CD71 or glycophorin A)

2. The mixture of labeled and unlabeled cells pass over a separation column, placed in the magnetic field of a MACS separator. Collect the flow through as the non-magnetic fraction

3. Remove the separation column from the magnet and flush out the retained cells as the positive selected cells

The main problem is maternal cell contamination that could be resolved with the future progresses:

- digital PCR is able to detect signals of contaminating maternal DNA
- microfluidic processor (single cell DNA analysis)
Biochip is based on differences in size and deformation characteristics. Nucleated fetal red blood cells range in diameter from 9 to 12 µm can deform and pass through a channel as small as 2.5 µm wide and 5 µm deep. Although adult red blood cells cannot deform and are retained by the 2.5 µm wide.

Schema of Biochip showing three rows of channels separating cells based on their size and ability to deform.
Considerations on non-invasive prenatal diagnosis:

In a recent national audit of the “British Society of Human Genetics”, was given a 45% reduction in invasive testing due to the increase in fetal sex determination performed using free fetal DNA in women requesting prenatal genetic testing for X-linked disorders

(Chitty et al. Prospective register of outcomes of free fetal DNA testing (PROOF) e results of the first year’s audit. Website:http://www.safenoe.org/meetings/march07/nipd)
Conclusions

• The discovery of free fetal DNA in maternal plasma is revolutionizing the field of prenatal diagnosis

• Advances in molecular technologies have enhanced the diagnostic applications of maternal plasma DNA analysis for non-invasive prenatal diagnosis

• High-throughput sequencing has been shown to be an effective tool for the detection of aneuploidies and in the future may be possible to **directly diagnose the mutation** responsible of the monogenic diseases

• PGD is now an established reproductive alternative to prenatal diagnosis, offered in quite a number of specialised centres in the world ([http://www.eshre.com](http://www.eshre.com))