

Genetics of Hemophilia

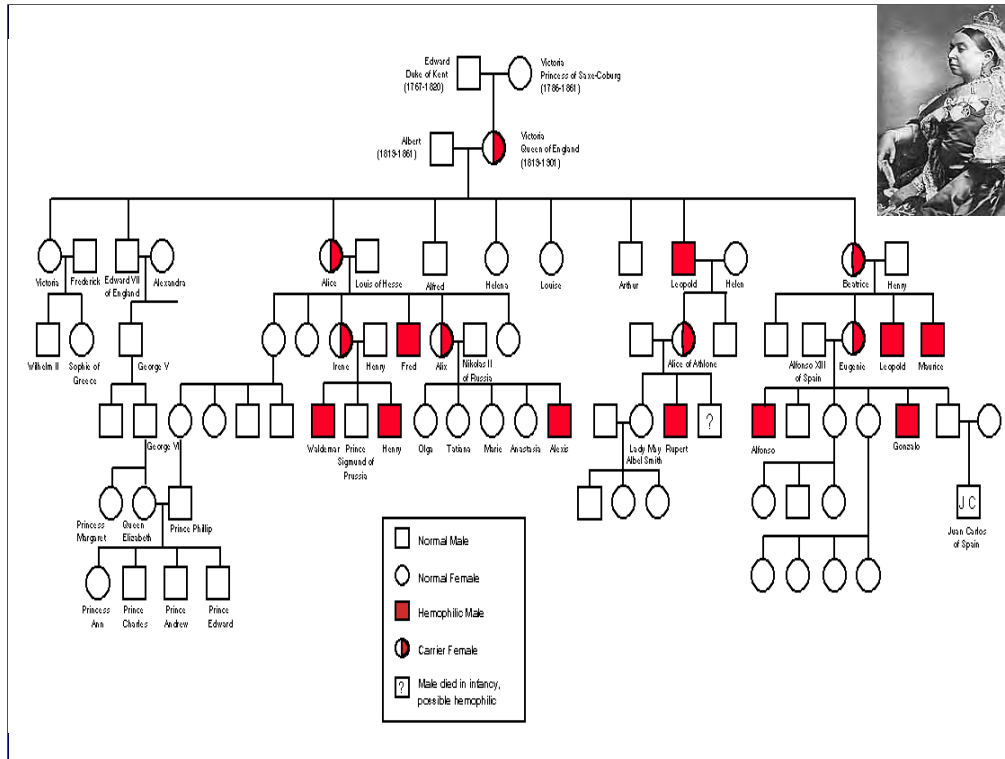
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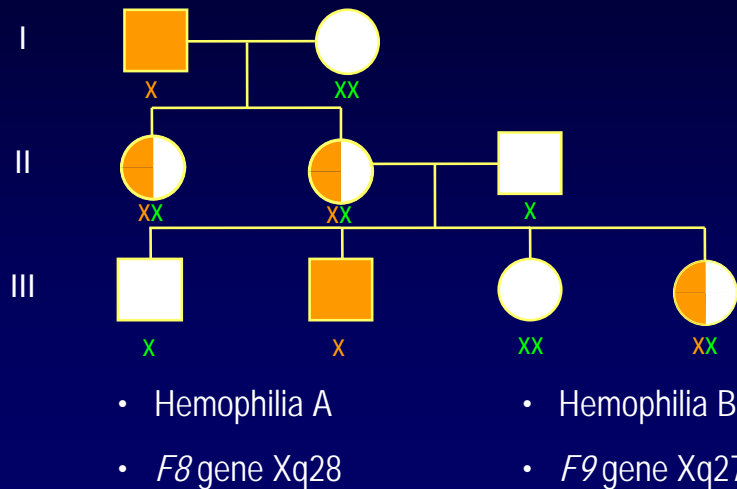
This presentation briefly introduces hemophilia and its inheritance pattern. It then describes the mutations responsible for hemophilia A and B and their analysis by mutation screening or DNA sequencing. The use of linkage analysis as an alternative means of genetic analysis is also described.

An algorithm for genetic testing is given, plus web-based and literature resources.



Pedigree of the most famous hemophilia family; that of Queen Victoria of the UK. The X-chromosome linked inheritance pattern of hemophilia is clearly seen, with females carrying the disorder, and males affected by hemophilia. Queen Victoria was the first known carrier in the family. The mutation responsible may have arisen in her father, Edward, Duke of Kent.

Hemophilia Inheritance – X-Linked Recessive



The defective gene in hemophilia A is *F8*, located at Xq28.

The defective gene in hemophilia B is *F9*, located at Xq27.

This pedigree shows the inheritance of the affected (orange) and normal (green) X chromosomes. Males have a single X, plus a Y chromosome, females have two Xs.

Males with hemophilia in the first and third generation share the same X chromosome. They have the same mutation and thus the same, or very similar hemophilia severity. Daughters of a male with hemophilia are all obligate carriers; they can only inherit a copy of the affected X chromosome from their father.

The third generation of the family demonstrates the four possible outcomes for children of the obligate carrier; unaffected or affected male, non-carrier or carrier female. Genetic analysis can be used to determine which X chromosome possible carriers have inherited.

Hemophilia Severity

- Hemophilia A <50% normal activity FVIII:C
- Hemophilia B <50% normal activity FIX:C
 - Severe <1% 40% of patients
 - Moderate 1-5% 10% of patients
 - Mild >5% 50% of patients

In all patients with hemophilia, except those with hemophilia B Leiden (later), hemophilia is a lifelong disorder of the same severity. In a family with hemophilia, the same mutation is inherited by all affected males, and the hemophilia severity in each of them will be very similar throughout their lives.

Hemophilia Symptoms

- Bleeding - prolonged, internal
- Severe disease
 - spontaneous joint and muscle bleeds
- Moderate disease
 - bleeds following minor trauma
- Mild disease
 - bleeds following major trauma

There is a strong correlation between residual clotting factor level and severity of bleeding symptoms. Spontaneous bleeding is only seen in severe disease. Affected males with severe disease are generally diagnosed by the age of one year, even where there is no prior family history of hemophilia.

In males with mild disease, it is not unusual for the disorder to not be diagnosed until middle age, possibly following prolonged bleeding at surgery. A history of bleeding episodes may then be recognized.

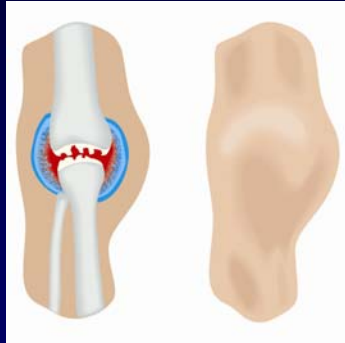
Hemophilia Symptoms



- Painful target joint
- Crippling arthropathy

Symptoms of hemophilia include bleeding into joints and muscles. Knees, ankles and elbows particularly are affected. Target joints develop when bleeding occurs recurrently into the same joint, causing progressive arthropathy.

Haemophilic Arthritis in a Knee Joint



© World Federation of Hemophilia - Hemophilia in Pictures

During a joint bleed, blood can fill the space normally filled with synovial fluid. Excessive bleeding can swell the joint. Resorption of blood can be associated with cartilage damage and eventually lead to cartilage loss. Arthritis due to progressive joint damage results in limited and painful joint movement.

Hemophilia Treatment and Annual Costs in the UK

- Severe disease - replacement clotting factor therapy required to stop bleeding (recombinant or plasma-derived), for 70kg male
 - Prophylaxis £160,000; on demand £100,000
 - Immune tolerance therapy (ITT) for hemophilia inhibitory antibody £300,000-800,000
- Mild disease - DDAVP provokes stimulated release of endogenous FVIII
 - DDAVP £100

Treatment available varies widely in different parts of the world. Current UK costs are shown.

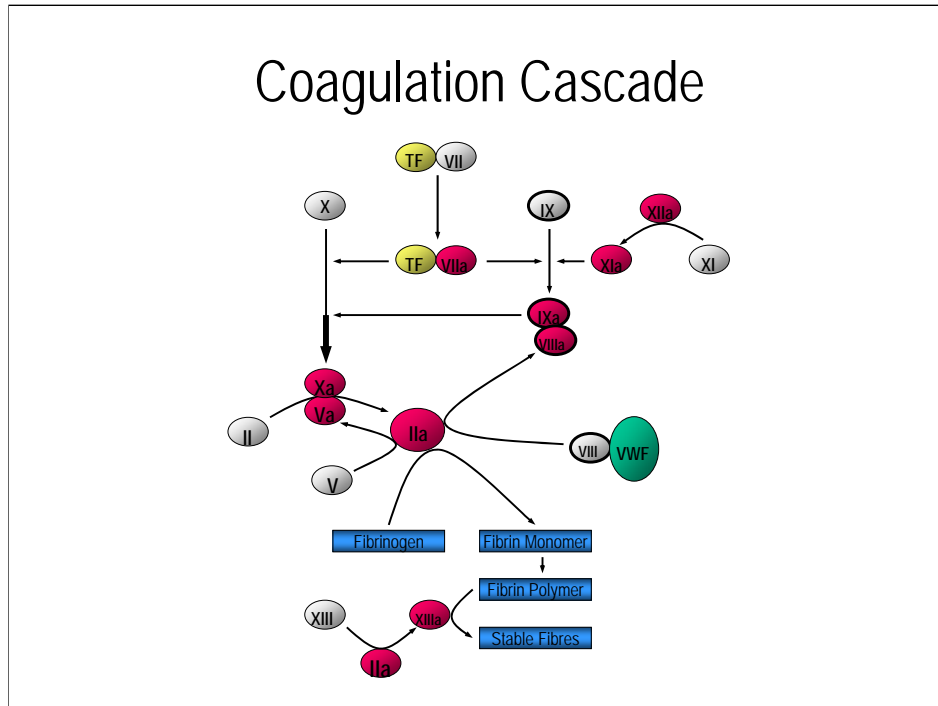
Hemophilia Prevalence

- X-chromosome linked inherited bleeding disorder
- 2 varieties
 - **hemophilia A**; 1 in 10,000 population
 - coagulation factor VIII deficiency

 - **hemophilia B**; 1 in 50,000 population
 - coagulation factor IX deficiency

Hemophilia occurs worldwide at very similar prevalence rates, as the mutations responsible recur due to intrinsic features of the *F8* and *F9* genes. Hemophilia A is five-fold more prevalent than hemophilia B.

Coagulation Cascade

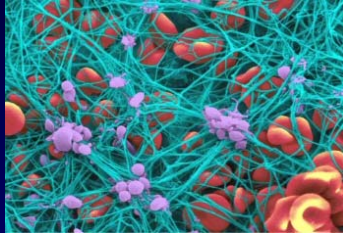


The Coagulation Cascade

Von Willebrand factor (vWF) carries and protects factor VIII (FVIII) from proteolytic degradation. Thrombin (FII) cleaves and activates FVIII to FVIIIa. FVIIIa then acts as a co-factor for activated FIXa, which activates FX to FXa. The eventual consequence of the cascade is to form stable cross-linked fibres of fibrin in blood clots. Insufficient FVIII or FIX results in less stable clots, due to ineffective fibrin formation.

Insufficient FVIII or FIX due to hemophilia A or B will result in the same symptoms of bleeding as the two coagulation factors are involved in the same stage of the cascade.

Fibrin Cross-Linked Blood Clot



(Credit: Yuri Veklich and John W. Weisel,
University of Pennsylvania School of Medicine)

Red blood cells trapped in a fibrin mesh of a normal blood clot are shown in this electron micrograph.

Coagulation Cascade

- FVIII is a co-factor for FIX in the activation of FX. Defects in either *F8* or *F9* result in the same symptoms as they function at the same stage of the coagulation cascade
- Treatment of hemophilia A and B patients differs. The correct factor must be replaced to prevent bleeding
- Male patients are (nearly) all diagnosed by specific coagulation tests

Coagulation testing is sufficient in almost all cases to diagnose hemophilia in affected males. Occasionally, cord blood from a new male baby from a hemophilia family is not suitable for coagulation factor level analysis due to clotting, so genetic testing is used to determine affected status. In a small proportion of cases, mild hemophilia A is not readily discriminated from type 2N von Willebrand disease by coagulation tests and genetic analysis is required instead.

Hemophilia carrier status in females is not readily determined by clotting factor levels. Genetic testing is necessary for accurate carrier status determination.

Mutations Responsible for Hemophilia A and B

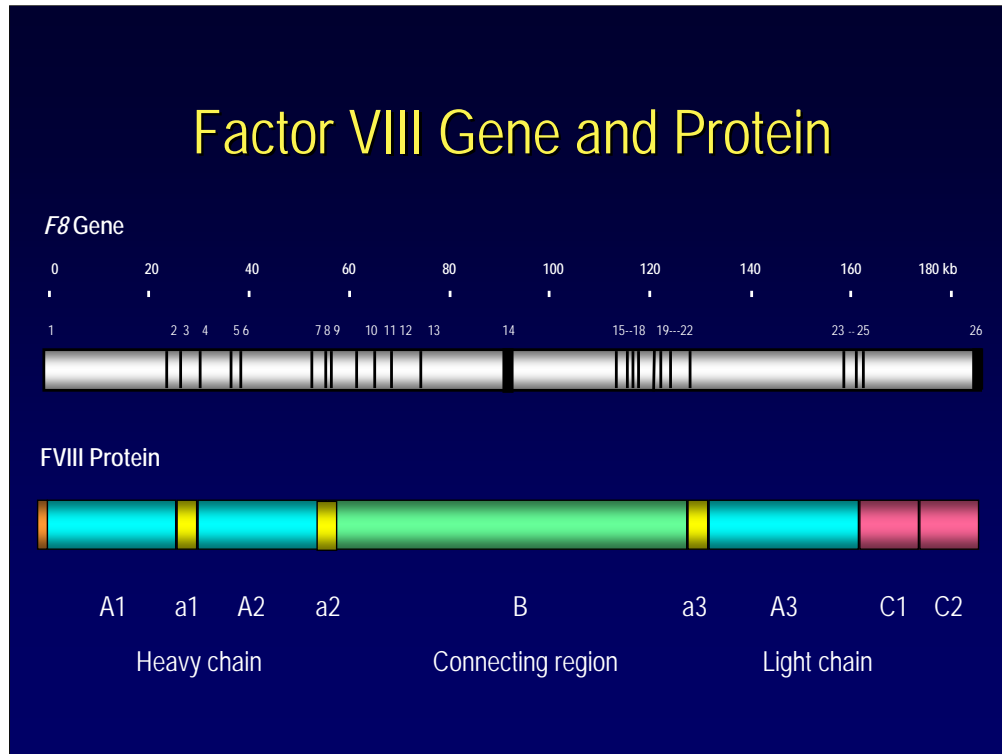
Glossary

F8 and *F9*: The factor VIII and IX genes

Factor VIII and IX: The coagulation factor proteins

Factor VIII:C (FVIII:C) and Factor IX:C (FIX:C): Coagulant activity

Factor VIII Gene and Protein

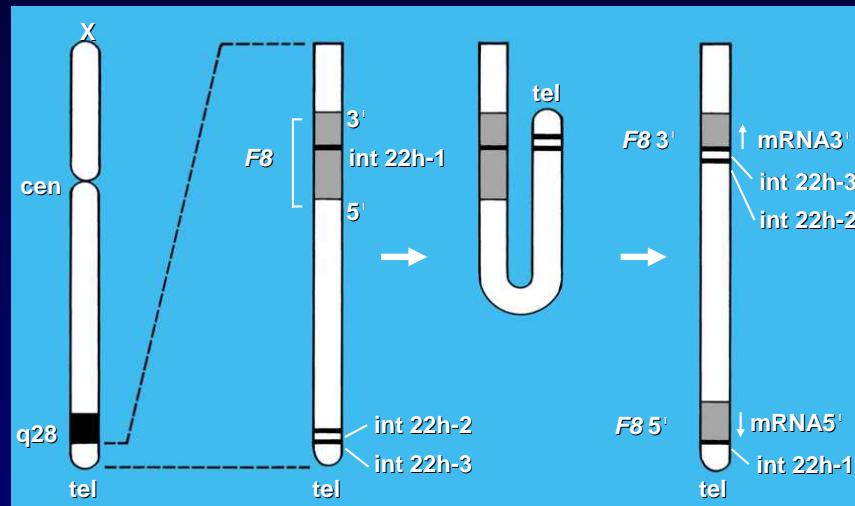


The upper panel shows the 186kb of genomic DNA of the *F8* gene. The gene has 26 exons and 25 introns. These encode an 8.8kb mRNA. The majority of exons are small, being at most a few hundred nucleotides in length. Exons 14 and 26 are rather larger, particularly exon 14, which is 3kb in length. Exon 14 encodes the B domain of the FVIII protein.

Introns 1 and 22 are notable for their large size and presence of repeated sequences, of which copies are present elsewhere on the X chromosome. These repeated regions are involved in the two intrachromosomal inversion mutations (below).

The FVIII protein has a repeated domain structure, with repeated A, a (acidic) and C domains, plus a single large B domain.

F8 Intron 22 Inversion



Based on a figure by Tuddenham EGD in Lancet 1994;343:307-8

Panel 1 shows the location of the *F8* gene at Xq28, towards the telomere (tel) of the long arm of the X chromosome.

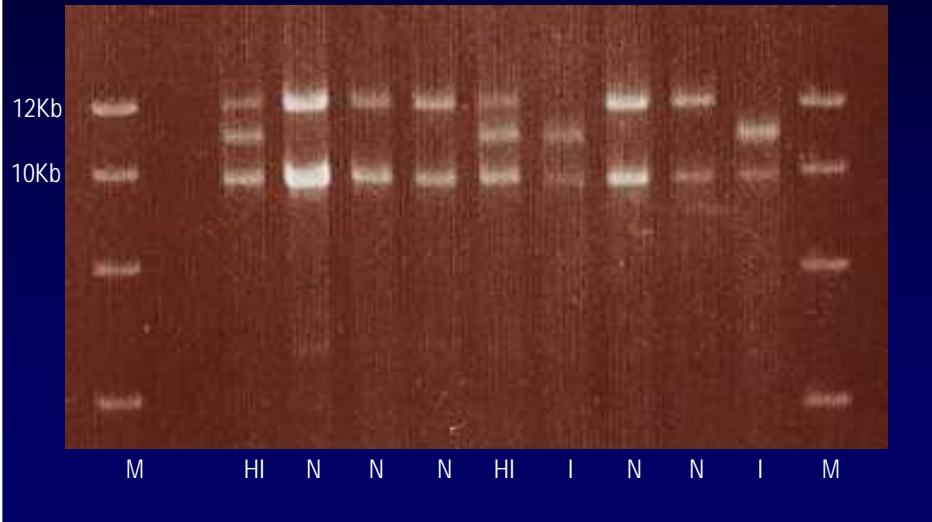
Panel 2 shows an expansion of this region. The *F8* gene is shown in grey. Intron 22 of the *F8* gene contains a region of 9.5kb (black bar), which is repeated twice approx 500kb 5' and telomeric to the gene. These regions are referred to as intron 22 homologous regions (int-22h) and are numbered 1-3.

Panel 3 shows looping round of the X chromosome, which occurs particularly at male meiosis. Two copies of int22h line up and homologous recombination can occur between them, as the sequences are >99% identical; only 5 nucleotides differ in 9.5kb between the three copies of the sequence. Recombination can occur with either of the two int22h copies, but is observed more frequently with the distal int22h-3 copy, than with the proximal int22h-2 copy.

Panel 4 shows the result of intrachromosomal recombination, or inversion. The 5' 22 exons of *F8* have been relocated to the telomeric end of the X chromosome, whilst the 3' four exons (23-26) remain in their original location. The *F8* gene is thus split into two parts, facing in opposite orientations, separated by approx 400kb of sequence. This split *F8* gene cannot encode functional FVIII protein; severe hemophilia A always results.

The mutation can occur *de novo* more frequently in males' single unpaired X chromosome. The status of a resulting carrier daughter is only apparent when she gives birth to an affected male, the grandson of the male in whose germ cells the mutation arose.

F8 Intron 22 Inversion Analysis



Agarose gel electrophoresis of polymerase chain reaction (PCR) products from the intron 22 long PCR demonstrating:

N: individuals lacking the intron 22 inversion

I: males hemizygous for the intron 22 inversion

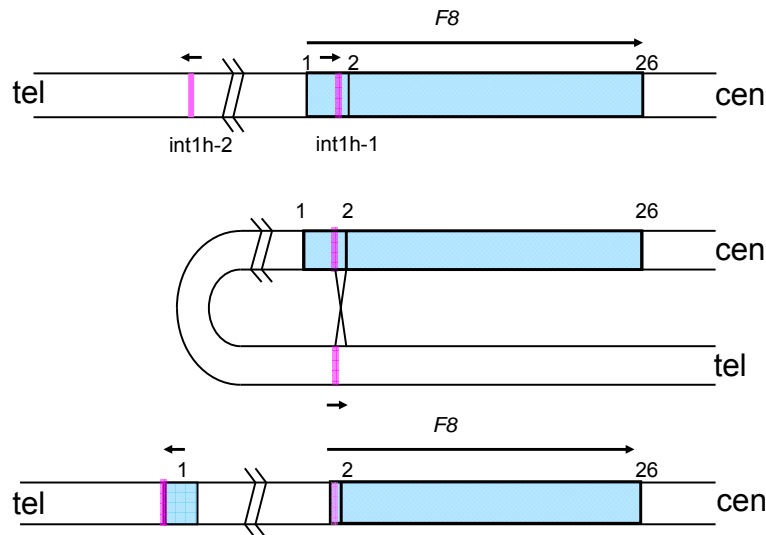
HI: females heterozygous for the intron 22 inversion

M: size marker

F8 Intron 22 Inversion

- Results from homologous intrachromosomal recombination
- Inversion mutation occurs *de novo* once per 10,000 male meioses
- Every ejaculate contains at least one sperm with a *F8* intron 22 inversion mutation
- Responsible for 45% of severe hemophilia A

F8 intron 1 inversion



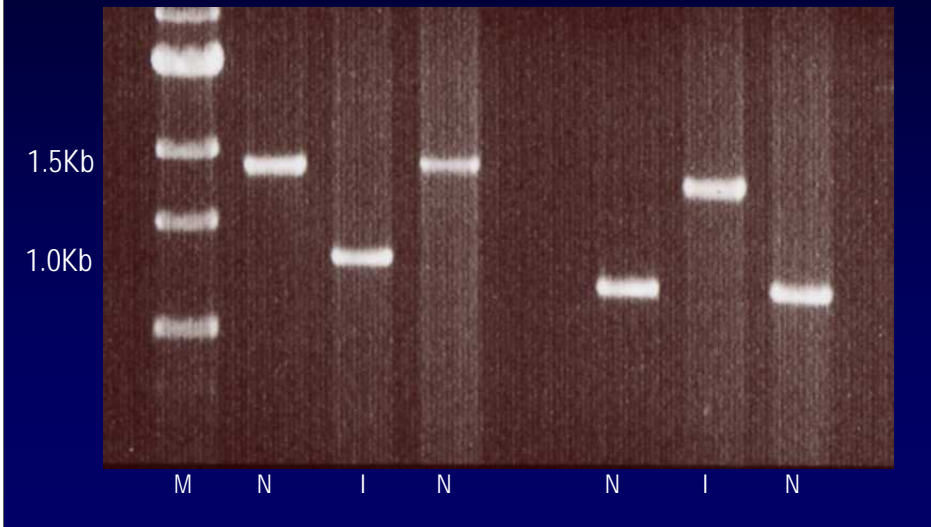
Based on a figure by Bagnall R et al in Blood 2002;99:168-174

Panel 1 shows the location of the *F8* gene at Xq28, towards the telomere (tel) of the long arm of the X chromosome. Intron 1 of *F8* has a 900bp region, repeated once, 5' and telomeric to *F8*, by approx 140kb.

Panel 2 shows looping round of the X chromosome, which probably occurs particularly at male meiosis, as for the intron 22 inversion. Two copies of int1h line up and homologous recombination can occur between them, as the sequences are >99% identical. Recombination is observed much less frequently with the int22 inversion. This is likely due to physical constraints for intron 1 inversion.

Panel 3 shows the result of intrachromosomal recombination, or inversion. The promoter and exon 1 of *F8* have been relocated to the telomeric end of the X chromosome, whilst the remainder of the *F8* gene stays in its original location. The *F8* gene is thus split into two parts, facing in opposite orientations, separated by approx 140kb of sequence. This split *F8* gene cannot encode functional FVIII protein; severe hemophilia A always results. Approx 2% of males with severe hemophilia A have this mutation.

F8 Intron 1 Inversion Analysis



Agarose gel electrophoresis of PCR products from the intron 1 PCR demonstrating:

N: hemizygous males (single X chromosome) lacking the intron 1 inversion

I: hemizygous male with the intron 1 inversion

M: size marker

F8 Intron 1 Inversion

- Similar to intron 22 inversion
- 900 bp region 5' to *F8* gene crosses over with homologous region in intron 1
- Results in *F8* gene lacking a promoter and first exon
- Responsible for approx 2% of severe hemophilia A

Intrachromosomal inversions cause
50% of cases of severe hemophilia A

Both inversions are seen at approximately the same frequency in all populations worldwide.

The intron 22 inversion is far more prevalent than the intron 1 inversion

This likely results from the greater distance of the extragenic int22h regions from *F8*, and the larger size of the homologous region (nearly 10kb, rather than the 1kb of int1h).

Examples of Point Mutation

-Cys Arg Lys Lys Thr Gln-	Normal
-TGC CGA AAA AAA ACG CAG -	sequence
-Tyr Arg Lys Lys Thr Gln-	Missense
-TAC CGA AAA AAA ACG CAG-	
-Val Stop	Nonsense
-GTC TGA AAA AAA ACG CAG-	
-Val Arg Lys Lys Arg Met-	Frameshift
-GTC CGA AAA AAA CGC AGT-	(eg A ₈ >A ₇)

The majority of non-inversion cases of hemophilia A, and nearly all cases of hemophilia B result from point mutations, affecting a single nucleotide. Examples of the most common types of mutation are shown above.

Panel 1 shows a normal (wild-type) nucleotide sequence and predicted amino acid sequence resulting from it.

Panel 2 shows a G>A nucleotide substitution, which predicts replacement of Cys by Tyr. Most hemophilia patients, with both hemophilia A and B, have a missense mutation. These alterations can be anywhere in the *F8* or *F9* genes. Disease severity depends on the location and any particular function of the amino acid affected.

Panel 3 shows a C>T substitution, which predicts replacement of Arg by a premature termination codon (stop or X). Nonsense mutations prevent any FVIII or FIX being made, so always result in severe hemophilia.

Panel 4 shows the loss of an A nucleotide from a run of A nucleotides. Such repetitive sequences are always prone to DNA replication errors, with a nucleotide more frequently being lost than gained. *F8* particularly has several runs of A nucleotides. Recurrent deletions (and insertions) are seen in each of these. They result in severe (and occasionally moderate) disease.

Other Mutation Types

- Deletion of part or all of gene (200bp to >200kb)
- Insertion into gene (repetitive sequence)
- Splicing error affecting production of mRNA

Up to 5% of patients with severe hemophilia have a deletion of all or part of the *F8* or *F9* gene. Deletions of one or a few exons are more common than whole gene deletions. No normal FVIII or IX can be produced in these individuals, so severe disease always results.

A very small number of patients have an insertion of a repetitive element (LINE1 or Alu) into the gene. This causes severe hemophilia by disrupting the coding sequence of the gene.

Point mutations can affect splicing of pre-mRNA to mature mRNA. There are two virtually invariant nucleotides at each end of each intron; GT at the 5' end (splice donor) and AG at the 3' end (splice acceptor). Alterations of these nucleotides will almost always disrupt splicing and cause severe (sometimes moderate) disease. Alteration of other nucleotides near the splice site can result in less severe disease. Exonic splice enhancer sites can also be altered by point mutation; some apparently silent (no amino acid substitution) mutations, for example in exon 11 of the *F8* gene, have been shown to disrupt splicing. Potential splice mutations can be predicted using web-based splice prediction software.

Ways to Eliminate FVIII Activity (severe disease)

- Intron 1 or 22 inversion
- Delete part of gene
- Insert extra nucleotides
- Nonsense mutation
- Splice site defect
- Missense mutation at strategic amino acid

Ways to Reduce FVIII Production (moderate/mild disease)

- Missense mutation, less important amino acid
- Splice site defect

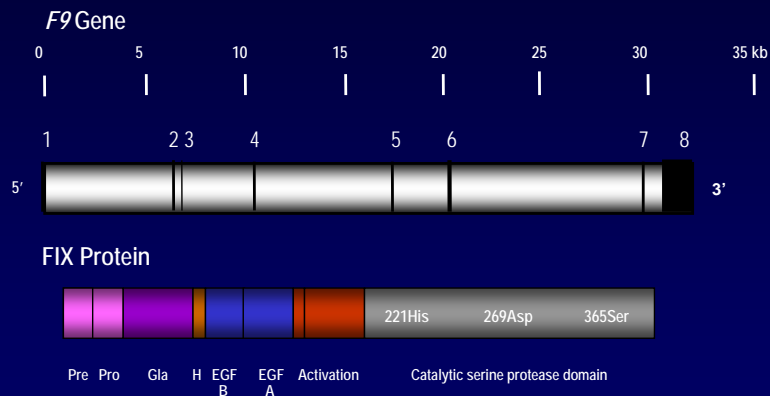
- Most families have a "private" mutation
- Mutation not identified in ~2% of patients

There is a good genotype-phenotype correlation in hemophilia. Many of the mutation types are always associated with severe disease. Virtually all mild and moderate hemophilia A patients have a missense mutation. Rarer patients have a splice site mutation.

In a small number of patients, despite mutation screening or DNA sequencing the entire coding region of *F8*, no mutation is identified. In those with severe disease, considerable work has been undertaken to try and identify other mutation locations, notably by Oldenburg and colleagues. Mutations deep in the introns are possible, but mRNA analysis has failed to identify mutations in these patients.

In moderate/mild disease, unless there is clear X-linked inheritance, type 2N von Willebrand disease is a possible cause of reduced FVIII levels. A VWF:FVIII:B assay can be undertaken on patients' plasma in such cases, and mutations sought in exons 18-25 of the VWF gene.

Factor IX Gene and Protein



The *F9* gene is considerably smaller than the *F8* gene, covering only ~35kb of genomic DNA. Its 8 exons encode a serine protease enzyme, responsible for cleavage of FX to FXa.

F9 Mutations

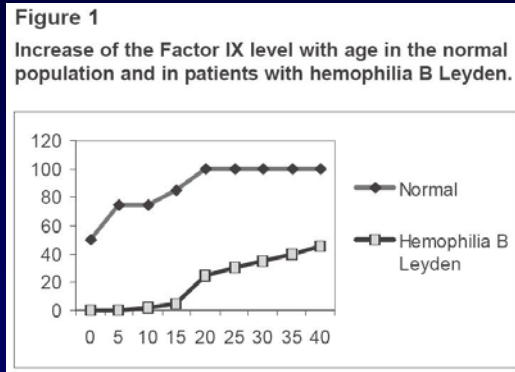
There are no mutations equivalent to the inversions seen in hemophilia A. The same general profile of non-inversion mutations is seen as in hemophilia A, with one important exception, hemophilia B Leiden. The majority of patients have point mutations, most resulting in missense mutations.

Hemophilia B Leiden

- Most hemophilia is lifelong disorder of same severity
- Small proportion of hemophilia B patients have FIX levels which increase at puberty
- “Hemophilia B Leiden”

This unusual form of hemophilia B is seen in approx 3% of hemophilia B patients. This is the only situation where hemophilia “gets better”.

Factor IX Levels in Normal Males and in Hemophilia B Leiden



© World Federation of Hemophilia - Mild Hemophilia (S Schulman 2006)

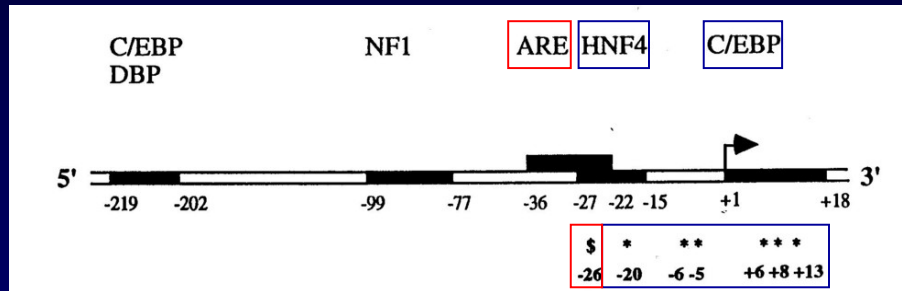
Hemophilia B Leiden results from specific *F9* promoter mutations

FIX levels in normal males demonstrate an age-dependent rise.

The FIX levels in a patient with hemophilia B Leiden prior to puberty were <1%. From puberty, an age-dependent rise mirroring that in normal males is seen. By adulthood, the majority of Leiden patients have FIX levels which just reach the normal range. In adults, bleeds and treatment are rare.

The baseline FIX level and the extent of the rise in level is dependent upon the *F9* mutation. These occur within a 35bp stretch of the *F9* promoter.

Transcription Factor Binding Sites in the *F9* Gene Promoter



Hemophilia B Leiden mutations affect the binding of transcription factors C/EBP or HNF4 to the promoter region. Individuals with nucleotide substitutions between -20 and +13, relative to the transcription start site, have been reported with this phenotype of hemophilia B. Following puberty, binding to the androgen response element starts to ameliorate hemophilia, as some *F9* transcription becomes possible.

Individuals with a point mutation at -26 (Brandenburg mutation) have lifelong hemophilia B. Before puberty, the mutation blocks binding to the HNF4 site. Following puberty, it blocks binding to the androgen response element.

Ways to Eliminate FIX Activity (severe disease)

- Delete part of gene
- Insert extra nucleotides
- Nonsense mutation
- Splice site defect
- Missense mutation at strategic amino acid
- Promoter mutation

Ways to Reduce FIX Production (moderate/mild disease)

- Missense mutation, less important amino acid
- Splice site defect
- Promoter mutation

- Most families have a "private" mutation
- Mutations detected in 99% of patients

There is a good genotype-phenotype correlation in hemophilia B. Many of the mutation types are always associated with severe disease, as for hemophilia A. Virtually all mild and moderate hemophilia B patients have a missense mutation. Rarer patients have a splice site mutation. Missense mutations are more common in hemophilia B than in hemophilia A.

In a very small number of patients, despite mutation screening or DNA sequencing the entire coding region of *F9*, no mutation is identified. This proportion is smaller than in hemophilia A. Deep intronic mutations are rare in hemophilia B.

Genetic Analysis Options in Hemophilia

1. Seek mutation in affected male, then use presence/absence of mutation to determine female carrier status and enable PND
2. Use linkage analysis to track affected allele around the family, without knowledge of the causative mutation

F8 or *F9* Gene Mutation Screen

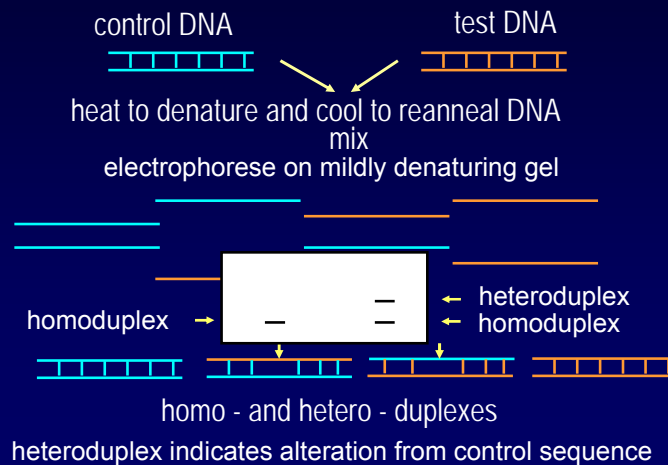
- Extract DNA from blood (white cells)
- PCR amplify exons & promoter
 - 30 PCR amplicons for *F8* (26 exons)
 - 10 PCR amplicons for *F9* (8 exons)
- Use mutation screening technique (CSGE, DHPLC, SSCP etc) or DNA sequence each amplicon to identify mutation
- Polymorphisms (neutral variation) also seen;
 - *F8*, 3 exon 14 and 1 intron 7 polymorphisms
 - *F9*, 1 exon 6 polymorphism

Mutation Analysis

A wide variety of techniques is available to screen the genes for mutations. Once an amplicon with altered mobility is identified, indicating presence of a mutation, this single amplicon can be sequenced to identify the mutation. Alternatively, the entire coding region can be directly analysed by sequencing. A single mutation responsible for hemophilia is generally identified.

In addition to sequence changes responsible for hemophilia, neutral polymorphic changes are seen in the coding region or close to it. Sequence alteration at the same position is identified frequently; it is not associated with hemophilia, and can just be noted in laboratory documentation.

DNA Heteroduplex Formation



Conformation sensitive gel electrophoresis (CSGE) is an example of heteroduplex analysis. Denaturing high performance liquid chromatography (DHPLC) is based on the same principle.

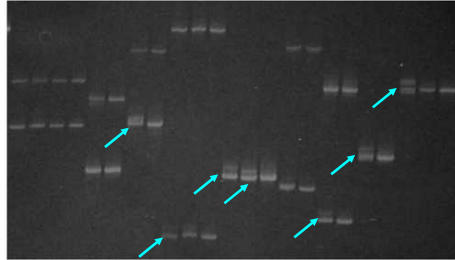
For males with hemophilia, having a single copy of the *F8* or *F9* gene, PCR amplified DNA for each separate amplicon needs to be mixed with that of a normal control male for the same amplicon, in equal proportions.

DNA is heated to 94°C for approx 5min to denature and separate the two DNA strands. It is then allowed to cool and reanneal at 65°C for approximately 30 min. Complementary sequences form homoduplexes; any sequence alteration in the patient's DNA in comparison with that of the normal control will result in a non-complementary base pair(s), which lacks hydrogen bonding. When electrophoresed on a mildly denaturing gel, heteroduplexes forms kinks or bends in the sequence, and electrophorese at a different rate from wild type sequence, generally being retarded. Following overnight electrophoresis, the gel is stained. Heteroduplexes are demonstrated by any difference in migration in comparison with wild type sequence.

The sensitivity of detecting sequence alterations is approximately 90%.

CSGE has recently been adapted to performance on automated capillary DNA sequencers as conformation sensitive capillary electrophoresis (CSCE).

Conformation Sensitive Gel Electrophoresis

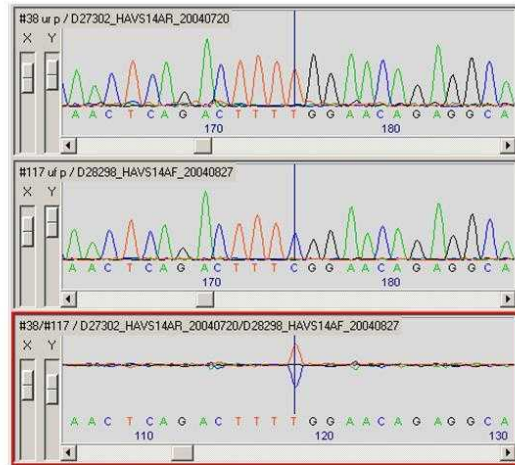


CSGE analysis of *F8* gene amplicons identifies several sequence alterations in heteroduplexed DNA

Conformation sensitive gel electrophoresis stained gel showing several different *F8* amplicons: Amplification for a number of the amplicons has been performed as a multiplex, where two amplicons have been amplified together. Up to 4 amplicons have been amplified together by a number of groups.

Arrowed amplicons show a migration shift, due to heteroduplex formation, indicating a sequence alteration in comparison with the control sequence. DNA sequencing will determine the nature of the alteration.

DNA Sequence Analysis



DNA sequence comparison of two patients to identify a sequence alteration using Staden sequence analysis software

Output from DNA sequence analysis can be analysed in a number of ways. If the whole coding region of the *F8* or *F9* gene is sequenced, sequence analysis software to aid the location of candidate mutations and polymorphisms simplifies the analysis.

In this example, the top two panels show a section of *F8* sequence trace for the same stretch of sequence in 2 individuals. The 3rd panel shows a trace difference, which subtracts one sequence from another, highlighting the difference between them, a C/T alteration in exon 14.

Mutation Analysis

- Sequence affected male's DNA
- Identify amplicon (1 of 30 for *F8*) with altered sequence
- Use reference (*F8*) sequence to interpret result of nucleotide change (eg missense mutation)
- Make judgement as to whether it is causative mutation in that patient (using mutation database, amino acid conservation etc)
- Seek mutation in suspected carrier females to determine carrier status



HAMSTeRS
The Haemophilia A Mutation,
Structure, Test, Resource Site.

<http://europium.csc.mrc.ac.uk>

The hemophilia A mutation, structure, test and resource site (HAMSTeRS) contains extensive information on *F8*. This includes lists of previously reported mutations and polymorphisms.

Additionally, amino acid alignment between 4 mammalian *F8* sequences enables simple determination of residues that are conserved across species.

CONDENSED TABLE OF FVIII POINT MUTATIONS: - MICROSOFT INTERNET EXPLORER

Exon No.	Codon No.	Original-mutated codon	Consequence	FVIII:C		
Exon 18	1966	CGA CAA (4)	Arg Gln	5-21	50	
Exon 18	1966	CGA CCA (1)	Arg Pro	3	?	M
Exon 19	1981	GGT GCT (1)	Gly Ala	<1	?	ε
Exon 19	1985	ACA AGA (1)	Thr Arg	?	?	
Exon 19	1987	GAA TAA (1)	Glu Stop	<1	?	ε
Exon 19	1988	ATG ATA (1)	Met Ile	14	31	
Exon 19	1997	CGG TGG (22)	Arg Trp	<1-5	4	Severe/l
Exon 19	1997	CGG CCG (2)	Arg Pro	<1	?	ε
Exon 19	1999	GAA GGA (2)	Glu Gly	1	?	ε
Exon 19	2003	GGC GAC (1)	Gly Asp	<1	20	ε
Exon 19	2009	GGG AGG (2)	Gly Arg	11-14	?	Mild
Exon 19	2011	AGC AAC (1)	Gly Asn	26	9	
Exon 19	2016	GTG GCG (5)	Val Ala	9-14	?	Mod
Exon 19	2017	TAC TGC (1)	Tyr Cys	3	5	M
Exon 19	2019	AAT AGT (3)	Asn Ser	5-20	5-13	Mod
Exon 20	2021	TGT TGA (1)	Cys Stop	<2	?	ε
Exon 20	2021	TGT TAT (1)	Cys Tyr	16	?	
Exon 20	2026	GGA GAA (1)	Gly Glu	8	?	
Exon 20	2026	GGA GTA (1)	Gly Val	<1	?	ε

Screenshot from HAMSTeRS, highlighting:

- 1) 2 different missense changes at codon 1966
- 2) Stop mutations resulting in severe hemophilia A (FVIII:C <1%)
- 3) A recurrent missense mutation at Arg1997Trp; 22 patients are recorded on the database with this mutation. It has been reported in severe to mild hemophilia (<1-5% FVIII:C). This may represent both intra-individual variation and assay variability.
- 4) Missense mutations affecting Gly residues at codon 2003 and 2009 result in differing hemophilia severities, reflecting possible different extent of structural alterations to the protein caused by these changes.
- 5) Missense mutations affecting Gly residue at codon 2026 result in differing hemophilia severities, reflecting possible different extent of structural alterations to the protein caused by each substitution.

FIX Home Page
http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html

FIX:C FIX:Ag Nt No. Nt chg CpG? Consequence

Münster 12	5		30970	C→G	N	283	S→R	Eigel & Horst	1937
GER 9689	6		30970	C→G	N	283	S→R	Wulff, Hermann et al	2348
France HB228	<1		30970	C→G	N	283	S→R	Goossens et al	2601
HB270			30972	A→G	N	284	Y→C	Double (see 31,328)	1048
HB 270	<5		30972	A→G	N	284	Y→C	Sommer et al	1048
HB816, Fr	<1	2	30973	C→A	N	284	Y→Stop	Ketterling, R. 1999	2217
HB203	1	<1	30973	C→A	N	284	Y→Stop	Tartary et al (1993)	1049
Besancon 7	1		30973	C→A	N	284	Y→Stop	Ketterling et al (1993)	1050
HB006	14		30980	C→A	N	287	P→T	Goossens et al	1984
GER 2234A	7		30981	C→A	N	287	P→H	Vidal et al (2000)	2653
es82	3	3	30981	C→A	N	287	P→H	Wulff et al (1995)	736
Unnamed	<1	<1	30981	C→T	N	287	P→L	Montejo et al (1999)	1857
GER 11606			30981	C→T	N	287	P→L	Chen et al (1991a)	251
9319	0	2	30984	T→C	N	288	I→T	Wulff, Hermann et al	2655
HD109	<1	<1	30985	T→G	N	289	I→M	Thompson (unpublished)	2760
GER 8892			30986	T→C	N	289	C→R	Dotseina et al (1991e)	519
PA 648	<1		30987	G→A	N	289	C→Y	Wulff & Hermann (1999)	1936
LY93	<1		30987	G→A	N	289	C→Y	Tagariello	2590
Balem 4	4		30987	G→C	N	289	C→S	Nagler et Vinciguerra	3054
UK 59	2		30987	G→T	N	289	C→F	Pestana et al	1488
HB162	<1		30987	G→T	N	289	C→F	Saad et al (1994)	736
Unnamed	<1		30987	G→T	N	289	C→F	Gastout et al (1993)	1051
Unnamed	<1		30987	G→T	N	289	C→F	Discoll et al (1996)	1487
Unnamed	<1		30987	G→T	N	289	C→F	Discoll et al (1996)	1646
Toulouse 4	<1		30987	G→T	N	289	C→F	Goossens et al	2637
GER 11629 (Ru)			30989	A1→CA	N	290	I→H	Wulff, Hermann et al	2656
UK 13	10		30992	G→A	N	291	A→T	Montandon et al (1999)	253
UK 33	7	19	30992	G→A	N	291	A→T	Green et al (1992a)	254
UK 41	9	11	30992	G→A	N	291	A→T	Green et al (1992a)	255
UK 71	7	10	30992	G→A	N	291	A→T	Saad et al (1994)	520
UK 239	10		30992	G→A	N	291	A→T	Saad et al (1994)	737
UK 249	6		30992	G→A	N	291	A→T	Saad et al (1994)	1052
UK 416			30992	G→A	N	291	A→T	Rowley et al	1499
HB 689	<1		30992	G→A	N	291	A→T	Li, X. 2000	2219
HB 690	1		30992	G→A	N	291	A→T	Li, X. 2000	2220
Oxford h2	2	3	30992	G→C	N	291	A→P	Winship & Dragon (1991)	282
France HB228	<1		30992	G→C	N	291	A→P	Double with 283 S→R (nucleotide 30970) Goossens et al	2601

A look at any page of the *F9* database will also demonstrate a number of reports of patients with the same mutation. Some are always reported in severe hemophilia B, for others a range of severities and residual FIX level is seen.

- 1) Stop mutation resulting in severe hemophilia B
- 2) Missense mutations at Pro287: 5 patients are recorded on the database. Mutations resulting in 3 different amino acid substitutions are recorded; T (Thr), H (His) and L (Leu). A range of severities dependent on the substitution is seen.
- 3) Missense mutations at Cys289: 9 patients are recorded on the database. Mutations resulting in 4 different amino acid substitutions are recorded; R (Arg), Y (Tyr), S (Ser) and F (Phe). A range of severities dependent on the substitution is seen.
- 4) Recurrent missense mutation affecting Ala residue at codon 291 result in differing hemophilia severities, severe to mild hemophilia (<1-10% FIX:C). This may represent both intra-individual variation and assay variability.

Any mutation identified should be compared with the database to determine whether it has been previously reported. If it has, a check should be made that the severity is consistent with that in previous patients.

Mutation Reporting

- Details of the gene analysed, the exon with the change, the nucleotide number and change identified, amino acid number and change (or other result of mutation) sent to referring clinician, ideally within 8 weeks of sample receipt
- If mutation is novel, justification of why it is considered a mutation
- Comment that the mutation can be sought in family members and that genetic counselling is appropriate

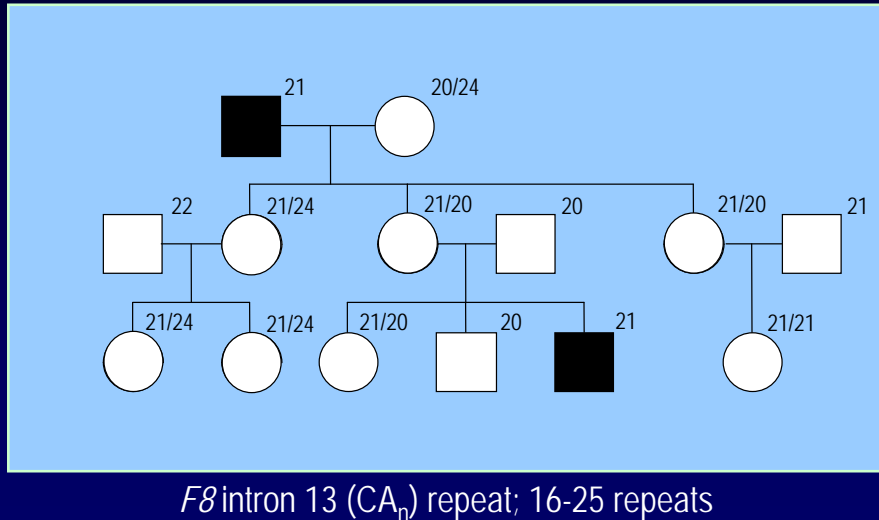
For reporting of mutations, a number of guidelines are available. It is recommended that these are followed.

The UK Clinical Molecular Genetics Society (CMGS) has produced recent guidelines on DNA sequencing, on report writing, and on hemophilia A and B genetic analysis. <http://www.cmgs.org/BPG/Default.htm>

The Human Genome Variation Society (HGVS) has extensive recommendations on a standardized system for documentation of nucleotide and amino acid sequence variation.

<http://www.genomic.unimelb.edu.au/mdi/rec.html>

Linkage Analysis in a Hemophilia A Family



A number of intragenic polymorphisms have been used for gene tracking in hemophilia. The *F8* gene has at least 4 short tandem repeat sequences (STR) that have been used, notably those in introns 13 and 22. It has a small number of well-characterized single nucleotide polymorphisms (SNP) that have also been used, most commonly that recognized by the restriction enzyme *Bcl* I in intron 18.

For linkage analysis to be informative, samples from a number of family members are required. Analysis of a male with hemophilia serves to “phase” of the disease; the allele associated with hemophilia in that particular family. In the above pedigree, carrier status of each of the females in generation 3 can be determined as each of their mothers is heterozygous for the intron 13 STR, and the origin of the 21 repeat allele, in phase with hemophilia in this family, can be determined.

The *F9* gene has no STRs, but has an insertion deletion sequence in intron 4, previously recognized by the *Dde* I restriction enzyme, plus approx 12 other well characterized and used SNPs. SNP prevalence varies extensively between different ethnic groups, and a population-specific panel requires selection for each patient population.

The HapMap project (<http://www.hapmap.org/>) has identified SNPs throughout the genome, and further useful SNPs not previously characterized for their utility in hemophilia genetic analysis are also accessible through various electronic databases, and may be useful in particular families.

The following slides give more detail on how linkage analysis can be used in hemophilia A and B families.

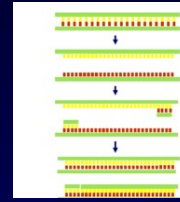
Linkage Analysis

- Technically simple
- Multiple members, including an affected member from the same family required
- Dependant on heterozygosity of key female relative(s)
- Ethnic variation in informativity
- In families with no prior hemophilia history, can only be used to exclude females as carriers

This slide discusses the advantages and disadvantages of linkage analysis in the genetic diagnosis of hemophilia. Linkage analysis is technically simple to perform as simpler methods are required than for mutation detection. These include PCR and restriction enzyme digestion followed by analysis of the DNA fragment sizes that result (previously referred to as RFLP — restriction fragment length polymorphism — analysis). Analysis can be performed using simple agarose or polyacrylamide gels. However, linkage analysis requires multiple members from the proband's family to be analysed to track the defective X chromosome's inheritance in the family. The success of this approach is also dependent on the informativity of the polymorphisms screened in the key female relative, and this can vary extensively between populations. This is particularly pronounced for polymorphisms in the *F9* gene. Linkage analysis therefore requires that the informativity of the various polymorphisms to be utilized in both *F8* and *F9* genes be established in the population being analysed, as there is a considerable ethnic variation in the heterozygosity rate for these polymorphisms. Despite these drawbacks, linkage analysis is the preferred approach for genetic diagnosis of hemophilia in developing countries.

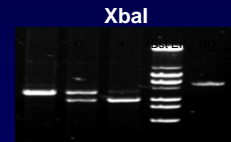
Approach for Linkage Analysis

- Polymerase chain reaction (PCR)



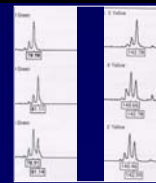
- Single nucleotide polymorphism recognized by restriction enzyme digestion (restriction fragment length polymorphism - RFLP)

_____ G _____
_____ A _____



- Repeat sequence polymorphisms (STRs or microsatellites) and variable number tandem repeats (VNTRs)

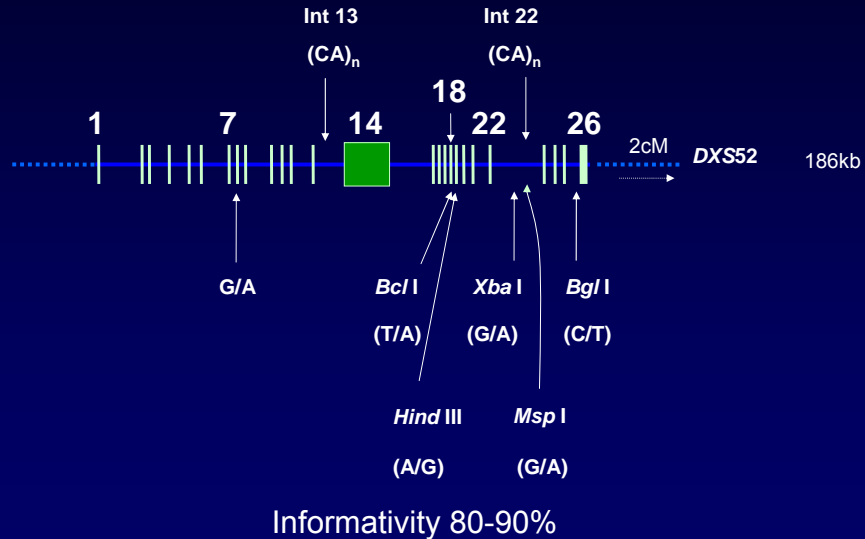
_____ CACACA _____
_____ CACACACA CA _____



Intron13 (CA)_n

This slide details the approach for linkage analysis. The target region surrounding the polymorphism is amplified by PCR. Bi-allelic SNPs can be genotyped commonly by restriction enzyme digestion followed by gel electrophoresis. Multi-allelic polymorphisms (STRs and VNTRs) can be genotyped by electrophoresis alone to determine PCR product size. This can be achieved by polyacrylamide gel electrophoresis and ethidium bromide or silver staining. Capillary electrophoresis using an automated DNA sequencer can also be used, if available.

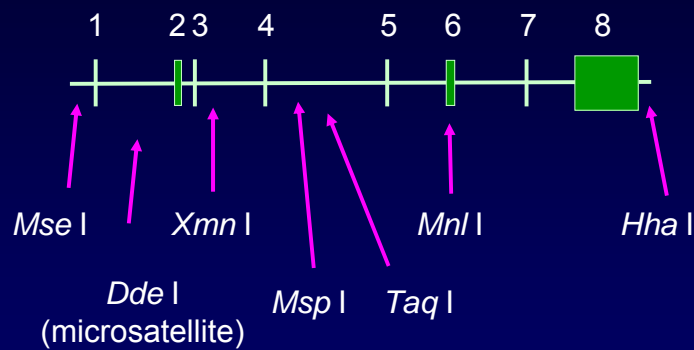
Commonly Used Polymorphic Markers in the *F8* Gene



This slide shows the eight frequently analysed polymorphisms in the *F8* gene (Peake et al, WHO Bull 1993). Of these, intron 7 G>A, *Bcl* I in intron 18, *Hind* III in intron 19, *Xba* I and *Msp* I in intron 22 and *Bgl* I in intron 22 are intragenic bi-allelic polymorphisms (SNPs). The intron 13 and intron 22 CA repeat polymorphisms are intragenic dinucleotide repeats. The combined informativity of all these polymorphisms can be up to 80-90% in some populations (Bowen DJ, Mol Pathol, 2002).

DXS52, an extragenic VNTR (also known as St14), is extragenic to the *F8* gene. At each meiosis, recombination between the polymorphism and the *F8* gene can occur, with a frequency of up to 5% of meioses. This marker should therefore only be used as a last resort, and then with extreme caution. If meiosis has separated the polymorphism from the *F8* gene, then a family may be given the wrong information regarding female carrier status or the hemophilia status of a male fetus.

Commonly Used Polymorphic Markers in the *F9* Gene



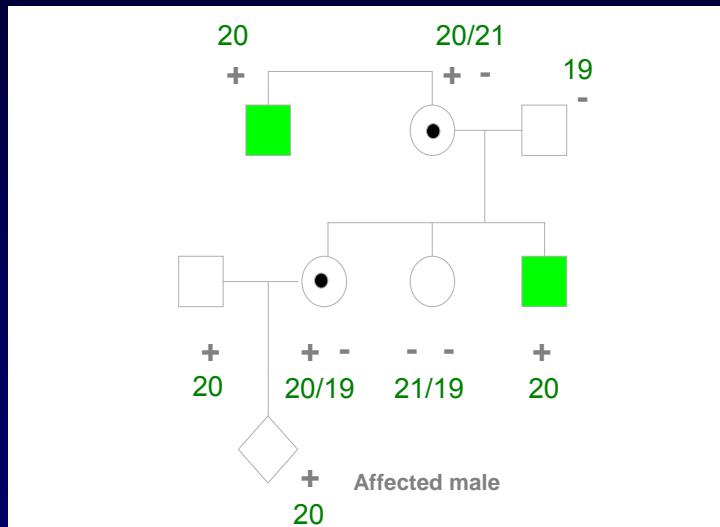
Combined informativity 80-90%

Many different polymorphisms have been identified in the *F9* gene and this slide details the main ones that have been used in linkage analysis. Most are SNPs recognized by PCR followed by restriction enzyme digestion and gel electrophoresis. *Dde*I comprises a number of 25bp repeat units, the number can vary between X chromosomes, and the two most common alleles differ in size by 2 repeats (50bp), although other sizes are more rarely seen. This useful polymorphism thus only requires PCR and gel electrophoresis to discriminate the different allele sizes.

For SNPs, the most useful ones have a heterozygosity rate close to 50%, which is the maximum possible heterozygosity. For STRs, the most useful have a >75% heterozygosity rate. The *F8* intron 13 and 22 STRs are generally less informative than this, dependent on the population being examined.

F9 polymorphisms differ widely in their informativity in different ethnic groups; those most useful in Caucasians may be rare or absent in Asian populations and vice versa. Combined marker informativity can reach 80-90%. (Bowen DJ, Mol Pathol, 2002)

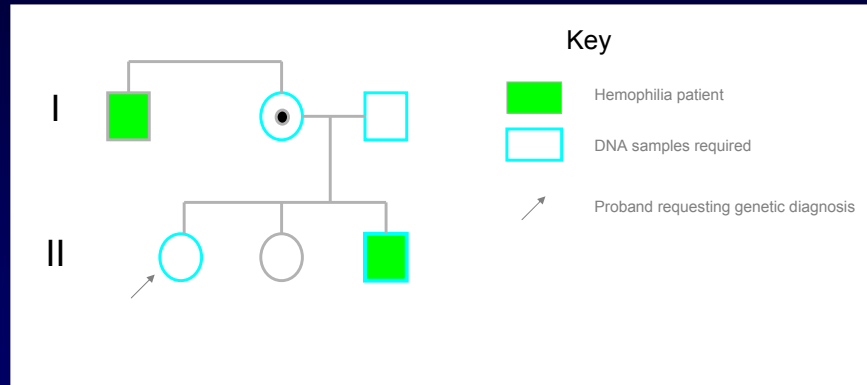
Interpretation of Linkage Analysis Results for Genetic Diagnosis



This slide demonstrates the interpretation of genotyping (both RFLP and microsatellite marker) results for linkage analysis. The defective X chromosome in this family segregates with 20 repeat of the intron 13 STR and the + allele for the *Bcl* I SNP. Carriers of hemophilia in this family have inherited this allele, whereas non-carriers lack this allele. The male fetus DNA (proband) shows 20 repeats for intron 13 and the + allele for *Bcl* I and is thus affected by hemophilia.

Fetal sex should be determined before any hemophilia-specific analysis is undertaken. Karyotyping undertaken by a cytogenetics laboratory is the preferred technique. If this is not available, fetal sex can be determined by PCR. XY chromosome-specific amelogenin PCR is often performed. *Amelogenin PCR* (Pugatsch *et al*, Bone marrow transp, 1996).

DNA Samples Required for Linkage Analysis



The female in generation II wants to know her hemophilia carrier status. From the pedigree structure, she has a 1 in 2 chance of being a carrier. Blood samples from which to extract DNA are required from both of her parents, the female requesting analysis, and her affected brother or another affected male relative, such as her maternal uncle.

The affected male is required to establish which allele is in phase with hemophilia in this family. The mother and father are required to enable the normal and the affected alleles inheritance to be tracked into the female requiring carrier analysis. The mother must be heterozygous for at least one marker for the process to be informative. The father's sample is required so that the allele that his daughter inherits from him can be determined.

Genetic Tests for Hemophilia

- *F8* intron 22 inversion
- *F8* intron 1 inversion
- *F8* screen in affected male
- *F9* screen in affected male
- Confirm / exclude mutation in ? carrier female (amplify single exon only)
- Linkage analysis
- Prenatal diagnosis

Each of the *F8* inversions can be examined using only one or 2 parallel PCR amplifications. Only males with severe hemophilia A and their female relatives are analysed.

Males with severe hemophilia A lacking an inversion mutation and all moderate and mild hemophilia A patients require all of their *F8* gene to be screened to identify a mutation. There are a number of locations where mutations are repeatedly found, and these can be targeted first.

For hemophilia B, there are no hotspots, and all 8 exons of *F9* should be analysed to seek a mutation. The exception is a patient in whom there is already a suspicion of the Leiden phenotype. In these patients, the promoter region should first be examined.

Ideally, an affected male should first be examined. In some families, these are unavailable. The next best option is an obligate carrier female (she is the daughter of an affected male or has 2 affected sons by different births, or has an affected son plus another affected male relative) as she should carry the familial defect. Once the familial mutation has been identified in one of these individuals, it can then be sought in any at-risk relatives by simply analysing one single *F8* or *F9* amplicon.

If mutation analysis is not available, or a mutation cannot be identified, or if the patient has a partial deletion of *F8* or *F9*, then linkage analysis can be used.

Prenatal diagnosis can be offered in a family known to have a mutation identified or who is known to be informative for linked polymorphic markers.

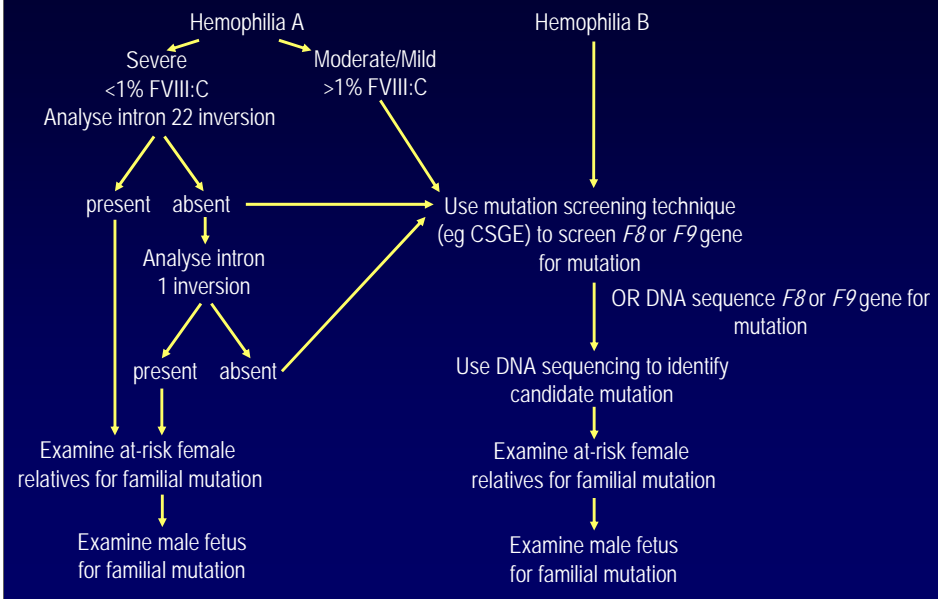
Prenatal Diagnosis (PND)

- 10-13 weeks gestation
- CVS biopsy
 - Karyotype
 - Check no chromosomal abnormalities
 - Determine sex
- If male, seek familial mutation. If female, report sex only
- Terminate if affected?

PND is generally only taken up by families with severe hemophilia. It is available to families in whom the causative mutation or an informative polymorphic marker has already been determined. Fetal gestation is checked by ultrasound analysis. At 10-13 weeks gestation, a sample of the developing placenta (chorionic villus) is taken. Biopsy cannot be performed prior to 10 weeks, as interference with the fetus may result in limb bud abnormalities. After 13 weeks, pregnancy termination requires induction of labour, whereas up to this date, it can be undertaken by suction evacuation, which is preferable for the woman undergoing the procedure.

Ultrasound guidance is used so that the edge of the placenta, avoiding the fetus, is sampled. A piece of tissue ~2 mm across is taken, using a needle inserted either transabdominally or transvaginally. This is examined by the cytogenetics laboratory to remove any maternal tissue from the biopsy (maternal contamination) and to analyse the chromosomes (karyotype). The fetus sample is analysed to check that there are no chromosomal abnormalities, and to determine sex, by examination of the two sex chromosomes. If the fetus is female, the family are informed and no further genetic analysis is performed (her carrier status can be determined later in life). If male, DNA is extracted from the biopsy and it is tested for the familial mutation or in phase polymorphic marker. Results of analysis should be available within 2-3 days of CVS sampling. The family then have the option to terminate an affected fetus if required.

Hemophilia Genetic Analysis Summary



Requests for Testing

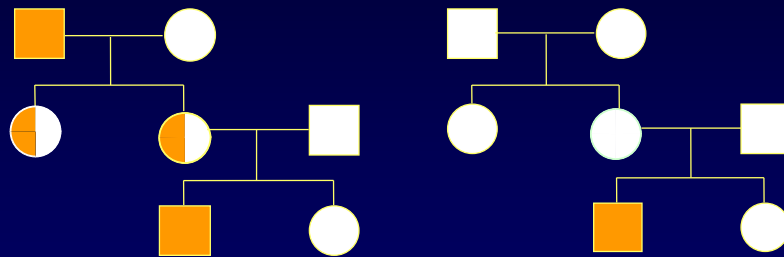
- All male patients
 - Determination of *F8/F9* mutation
- Familial hemophilia
 - Planned carrier testing of at-risk females from ~ age 12
- Sporadic hemophilia
 - Carrier testing of at-risk females following diagnosis of first male
- Difficult cases
 - *F8* screen to discriminate FVIII / VWF deficiency
 - Explain low FVIII / IX in females
- PND

The list describes the main individuals in whom mutations are sought. The WHO recommends that the mutation responsible for hemophilia is sought in all affected males, so that the information is available to female relatives requiring carrier status determination.

In some males, mild hemophilia A and VWD can be difficult to discriminate. A screen of the *F8* gene and identification of a mutation can clearly diagnose mild hemophilia A.

Females may have a low FVIII/IX level and experience some bleeding symptoms that have highlighted the low level, but have no male relatives affected by hemophilia. An examination of the *F8* or *F9* gene and identification of heterozygosity for a mutation can identify the cause of the deficiency.

New Mutations in Hemophilia



Family history of hemophilia

~ 60% families

Sporadic hemophilia

~ 40% families

The majority of families with hemophilia have a prior history of the disease, with more than one affected male in the family. Daughters of affected males are obligate carriers. The only X chromosome that they can inherit from their father carries a mutation. In such families, known to the hemophilia centre, families can be prompted to inform at-risk females that carrier analysis is available. Ideally, carrier analysis can be undertaken when females are mature enough to understand the analysis process and the meaning of their results, but prior to becoming sexually active.

Approx 40% of families have a single affected male and no prior family history of hemophilia. The origin of the mutation in such families may be in the hemophiliac himself, but more frequently, his mother is a carrier. The origin of new mutations is most frequently the unaffected maternal grandfather of the hemophiliac. In such families, the time of requesting genetic analysis often depends on hemophilia severity. If the affected male has severe hemophilia, he will generally be diagnosed by ~ 1 year old. His mother or maternal aunt(s) are often either pregnant or contemplating (further) children. Several requests for genetic analysis from females of childbearing age often result.

If the hemophilia is mild, the affected male may not be diagnosed until mid-life, possibly as a result of surgery or trauma. The family structure may then differ, but a number of requests from female relatives for carrier status determination may result following his diagnosis.

Mutation Survival - Severe Hemophilia

- Until recently, patients with severe hemophilia had shortened life expectancy and low reproductive fitness
 - mutations tended to be lost with the patients' death
 - new mutations more common in families with severe hemophilia

In severe hemophilia A and B, until very recently, affected males frequently did not live to reproduce. Mutations were not propagated extensively through their families. New mutations were common in severe hemophilia.

There are several mutation-prone locations in the *F8* and *F9* genes. These include CpG dinucleotides, which have a higher mutation rate than any other dinucleotide pair. Typically, the C is demethylated to result in a T nucleotide (C>T substitution). In *F8*, runs of A nucleotides are prone to deletions or insertions of a single nucleotide.

Mutation Survival – Moderate/Mild Hemophilia

- Mild hemophilia patients had longer lifespan and higher reproductive fitness
 - mutations tend to persist in the population
 - many families share the same mutation due to a founder effect
 - Local common mutations eg Arg1966Gln and Arg583Gly in *F8*

In mild and moderate hemophilia A and B, the same mutations are seen repeatedly, as they survive within the population. Males with the mutation live to reproduce and thus the mutation is propagated to further generations. Local founder effects are often seen, such that each locale may have its own common mutation, which is different from those in other locations.

Females with Hemophilia

- Some female hemophilia carriers experience bleeding problems
- Early in embryogenesis, one X chromosome is inactivated in all female cells; "Lyonization"
- Process is random
- May result in unequal inactivation of X chromosomes
 - Carriers with hemophilia
 - Carriers with normal FVIII/IX levels

The FVIII:C and FIX:C levels in female hemophilia carriers are highly variable. Up to 10% of female carriers have clotting factor levels lower than 50% and may experience bleeding in some situations. Occasional carriers of severe disease, like their affected male relatives, lack FVIII:C or FIX:C. Females known to be carriers can also have completely normal or even elevated levels of FVIII:C or FIX:C.

Early in embryogenesis, when the fetus is at or around the 8-32 cell stage of growth, one X chromosome is inactivated by methylation in every female cell, so that all male and female cells have the same dosage of genes expressed from the X chromosome. This process, which is random, is described as Lyonization, after Mary Lyon who first described the process. All progeny cells maintain the same pattern of X chromosome inactivation throughout life. Any unequal inactivation of the two X chromosomes in a hemophilia carrier can result in either a female who bleeds (normal X has been inactivated more than the one carrying a mutation), or a carrier with a completely normal clotting factor level (X chromosome carrying a mutation inactivated to a greater extent).

Hemophilia Web Resources

- Hemophilia A web page "HAMSTeRS"
<http://europium.csc.mrc.ac.uk/WebPages/Main/main.htm>
- Hemophilia B web page
<http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html>
- Best Practice guidelines
<http://www.cmqs.org/BPG/Guidelines/2004/HemophiliaA.htm>
<http://www.cmqs.org/BPG/Guidelines/2004/HemophiliaB.htm>
- About hemophilia
<http://www.hemophilia.org.uk/>
<http://www.zlbehring.co.uk/zb/n26942/PFFAQs.htm>

Review Articles and Papers

- Graw J, et al. Hemophilia A: from mutation analysis to new therapies. *Nat Rev Genet.* 2005, 6:488-501
- Keeney S, et al. The molecular analysis of hemophilia A: a guideline from the UK hemophilia centre doctors' organization hemophilia genetics laboratory network. *Hemophilia.* 2005,11:387-97
- Mitchell M, et al. The molecular analysis of hemophilia B: a guideline from the UK hemophilia centre doctors' organization hemophilia genetics laboratory network. *Hemophilia.* 2005, 11:398-404
- Oldenburg J et al. Molecular basis of hemophilia A. *Hemophilia.* 2004, 4:133-9
- Lavery S. Preimplantation genetic diagnosis: new reproductive options for carriers of hemophilia. *Hemophilia.* 2004, Suppl 4:126-32
- Bolton-Maggs and Pasi, Hemophilias A and B *Lancet* 2003, 361:1801-09

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