Genome Editing and Engineering
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Module - 08
Applications of genome editing in treating human diseases
Lecture - 32
Human cell engineering in diseases: Hemophilia - Part A

Welcome to my course on Genome Editing in Engineering, in this module we have been discussing about the Applications of genome editing in treating human diseases. In today's lecture we will be discussing about Hemophilia. We will start with some basic concepts regarding the disease and existing treatment modalities and slowly move on to the applications of gene therapy and then finally genome editing in the treatment of hemophilia.

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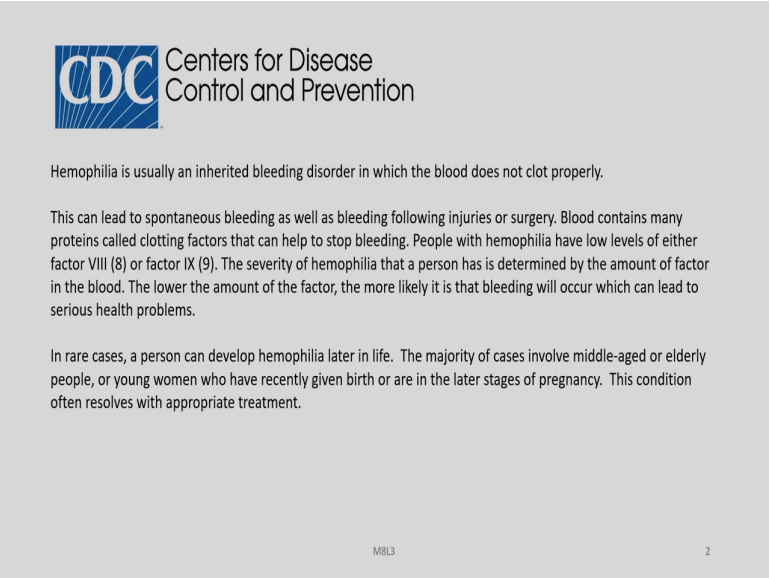

So, center for disease control and prevention describes hemophilia as an inherited bleeding disorder in which the blood does not clot properly and due to this non-clotting of the blood the patient can bleed and due to various factors like injuries or even during surgery. Blood contains many proteins called clotting factors that can help to stop bleeding, people with hemophilia have low levels of either factor VIII or factor IX, the severity of hemophilia that a person has is determined by the amount of factor in the blood.

The lower the amount of the factor the more likely it is that bleeding will occur which can lead to serious health problems. In rare cases a person can develop hemophilia later in life, the majority of cases involve middle aged or elderly people or young women who have recently given birth or are in the later stages of pregnancy; this condition often resolves with appropriate treatment.

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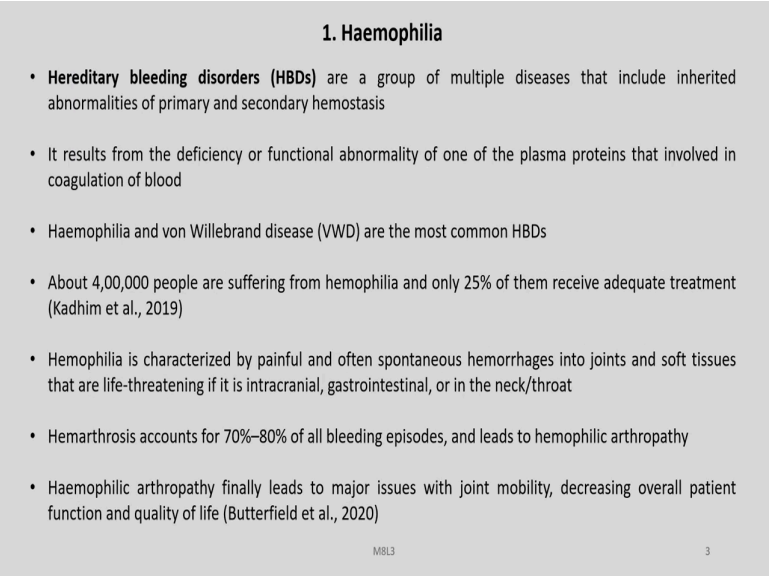

So, we now know that hemophilia is a hereditary bleeding disorder. In fact, hemophilia is a group of multiple diseases that include inherited abnormalities of primary and secondary hemostasis, it results from the deficiency of functional abnormality of one of the plasma proteins that in that is involved in the coagulation of blood. Haemophilia and von Willebrand disease VWD are the most common hereditary bleeding disorders. About 400000 people are suffering from hemophilia and only 25 percent of them receive adequate treatment.

The hemophilia disease is characterized by painful and often spontaneous hemorrhages into joints and soft tissues, there are life threatening if it is intracranial, gastrointestinal, or in the neck or throat. Hemarthrosis accounts for 70 to 80 percent all of all bleeding episodes and leads to hemophilic arthropathy. Hemophilic arthropathy finally leads to major issues with joint mobility decreasing overall patient function and quality of life.

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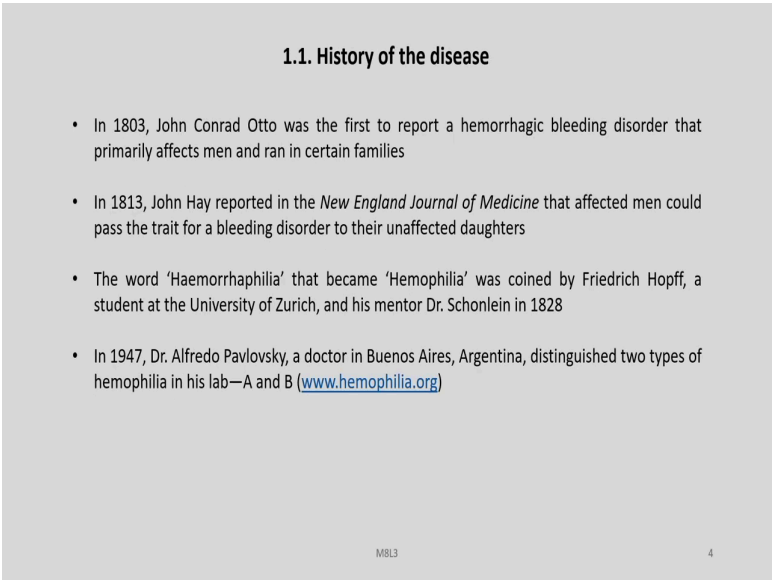

Let us go into the history of these disease. In 1803, John Conrad Otto was the first to report a hemorrhagic bleeding disorder that primarily affects men and ran in certain families. In 1813, John hay reported in the New England Journal of medicine that this disease affected men could pass the trait for a bleeding disorder to their unaffected daughters. The word Haemorrhaphilia that became Hemophilia was coined by Friedrich Hopff, a student at the university of Zurich and his mentor Dr. Schonlein in 1823.

In 1947 Dr. Alfredo Pavlovsky a doctor in Buenos Aires Argentina distinguished 2 types of hemophilia in his lab called as hemophilia A and hemophilia B. This website hemophilia.org contains many useful information regarding the disease. Let us look into the genetics of these particular disease as you can see in 1813 itself John Hay had reported about affected fathers passing on the disease to unaffected daughters.

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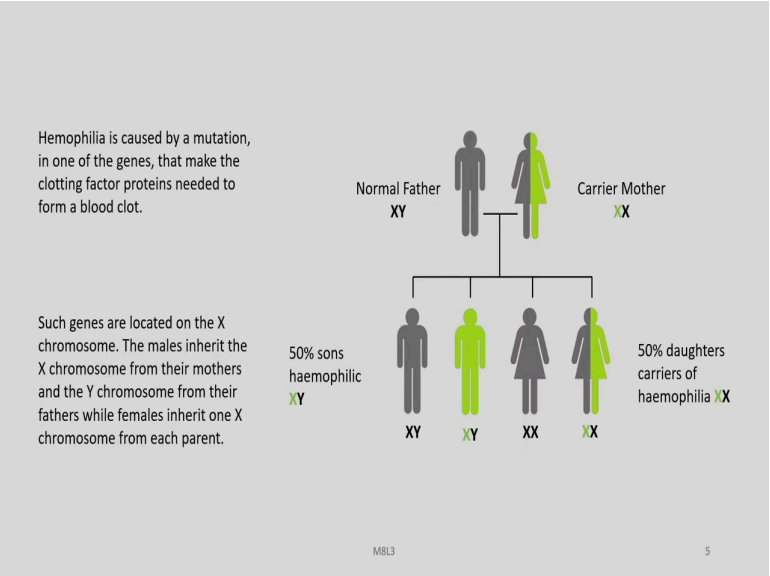

So, in these slide we are going to discuss about the inheritance of this disease or the genetics. Hemophilia is caused by a mutation in one of the genes that make the clotting factor proteins needed to form a blood clot and these genes are located on the X chromosome and we know the males inherit the X chromosomes from their mother and the Y chromosome from their father. While the daughters inherit the X chromosome one copy each from father and mother respectively.

Now, supposingly there is a normal father with XY normal chromosomes, but there is a carrier mother having a X chromosome which carries the mutated genes and one normal X chromosome. So, the off springs now would inherit in the pattern as shown in these picture. So, with this male who inherits the X chromosome from the father and sorry the X chromosome this individual male who inherits the X chromosome from the mother and the Y chromosome from the father will be a normal individual.

While this male offspring which inherits the X chromosome from the mother and the Y chromosome from the father will be having the disease, these female offspring inheriting the normal X chromosomes from mother and father respectively will be a normal individual. While the last female offspring inheriting the normal X chromosome from the father and the X chromosome carrying the mutated genes will be a carrier.

So, if you look into the overall distribution of the offspring between a normal father and a carrier mother 50 percent of the male off springs will be hemophilic, while 50 percent of the

daughters will be carriers and overall 50 percent of the children will be normal children. So, this is in brief, the genetics of the hemophilia disease. So, we will try to find out a little bit more information about these genes which are located on the X axis.

One may question why these individual having a mutated X chromosome is having the disease, the reason is the dominance of the X factors on the X chromosome against which there is no any corresponding genes on the Y chromosome.

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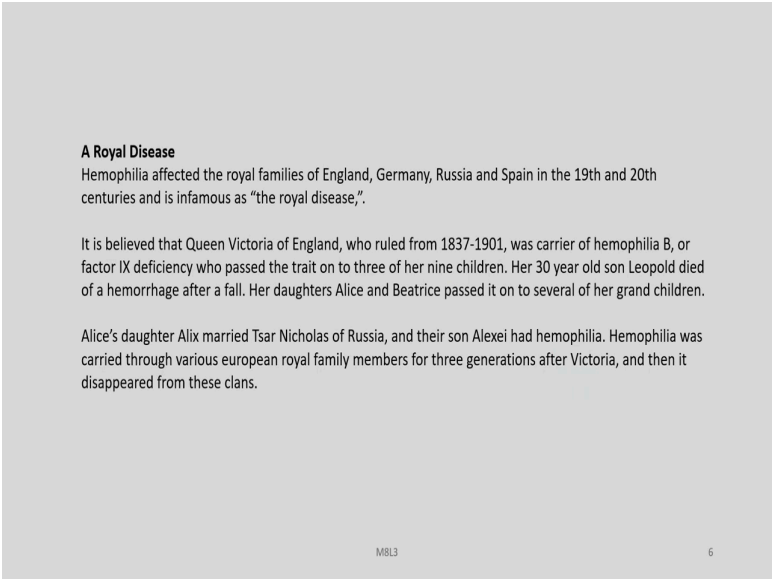

Hemophilia is also called as the Royal disease, this disease has affected the royal families of many European nations in the past like England Germany Russia and Spain in the 19th and 20th centuries and is therefore known infamously as the Royal disease.

It is believed that Queen Victoria of England who ruled from 1837 to 1901 was the a carrier of hemophilia B or factor IX deficiency who passed the trait onto 3 of her 9 children her 30 year old son Leopold died of a hemorrhage after a fall, her daughters Alice and Beatrice passed it on to several of her grandchildren. Alice Marry married John Nicholas of sorry Alice's daughter Alix married Tsar Nicholas of Russia.

And their son Alexei had hemophilia, hemophilia was carried through various European royal family members for 3 generations after Queen Victoria and then it suddenly disappeared from these royal families.

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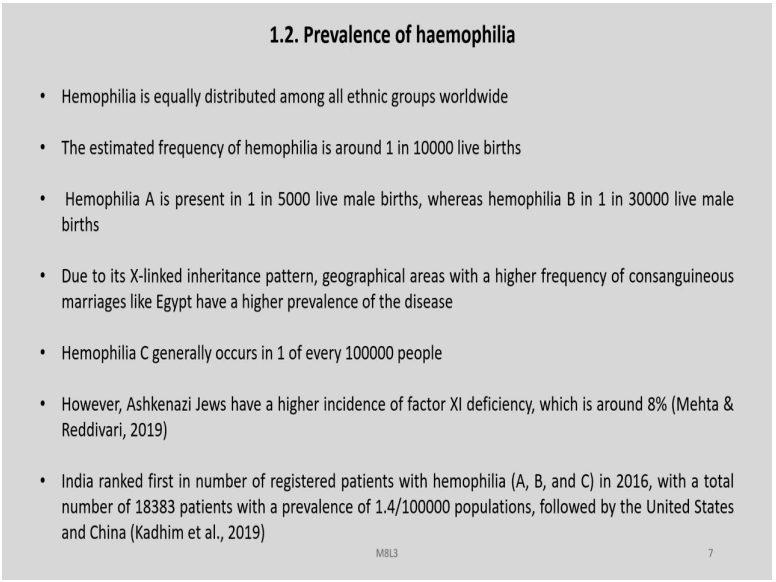

What is the prevalence of hemophilia apart from the infamous occurrence in the royal families of Europe, hemophilia is equally distributed among all ethnic groups worldwide the estimated frequency of hemophilia is around 1 in 100000 live births.

Hemophilia A is present in 1 in 5000 live male births; whereas, a hemophilia B is present in 1 in 30000 life male births. Due to it is X linked inheritance pattern geographical areas with a higher frequency of consanguineous marriages like Egypt have a higher prevalence of the disease. Hemophilia C generally occurs in 1 of every 100000 people.

However, Ashkenazi Jews have a higher incidence of factor IX deficiency which is around VIII percent. India ranked first in number of registered patients with hemophilia including A B C in 2017 with a total number of 18383 patients with a prevalence of a 1.4 in 100000 populations followed by the United States and China.

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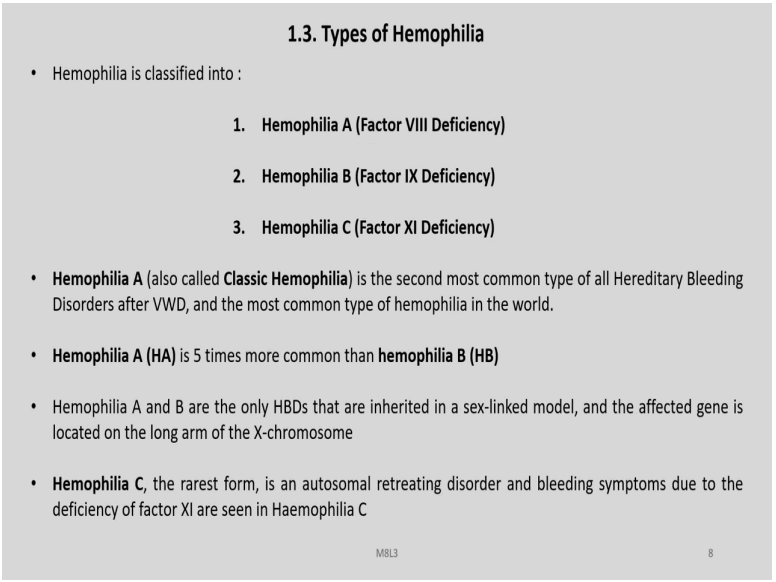

By this time we have known that there are 3 types of hemophilia, hemophilia A which occurs due to factor VIII deficiency, hemophilia B which occurs due to factor IX deficiency and there is a hemophilia C which is due to factor XI deficiency. Hemophilia A is also called Classic Hemophilia is the second most common type of all hereditary bleeding disorders after VWD and the most common type of hemophilia in the world. Hemophilia A or HA is 5 times more common than hemophilia B or HB.

Hemophilia A and B are the only HBDs that are inherited in a sex linked model and the affected gene is located on the long arm of the X chromosome. Hemophilia C is the rarest form. It is an autosomal retreating disorder and bleeding symptoms due to the deficiency of factor IX are seen in the hemophilia C cases.

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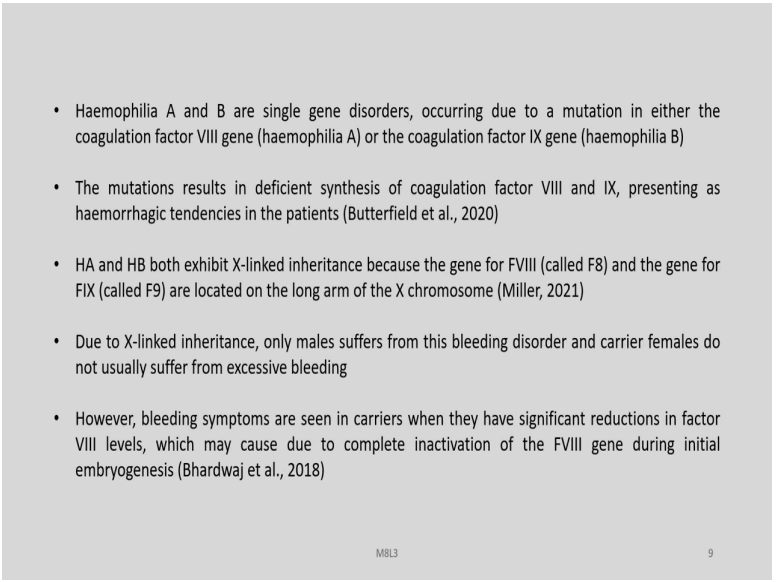

HA and HB are singled in gene disorders occurring due to a mutation in either the coagulation factor VIII gene or the coagulation factor IX gene and being single gene disorders. These this is the ideal candidate for gene therapy or by/through gene editing or gene engineering technologies.

The mutations result in deficient synthesis of coagulation factors VIII and IX respectively for HA and HB presenting as hemorrhagic tendencies in the patients; HA and HB both exhibit X link inheritance because the gene for factor VIII and the gene for factor IX are located on the long arm of the X chromosomes and there is no any corresponding chromosomal location on the Y axis. Due to X link inheritance only males suffer from this bleeding disorder.

And the carrier females do not usually suffer from the excessive bleeding, because the corresponding X chromosome, X as a buffer or you know dominating factor against these mutations in the long arm. However, bleeding symptoms are seen in carriers when they have sufficient significant reductions in factor VIII levels which may cause due to incomplete complete inactivation of the factor VIII gene during initial embryogenesis.

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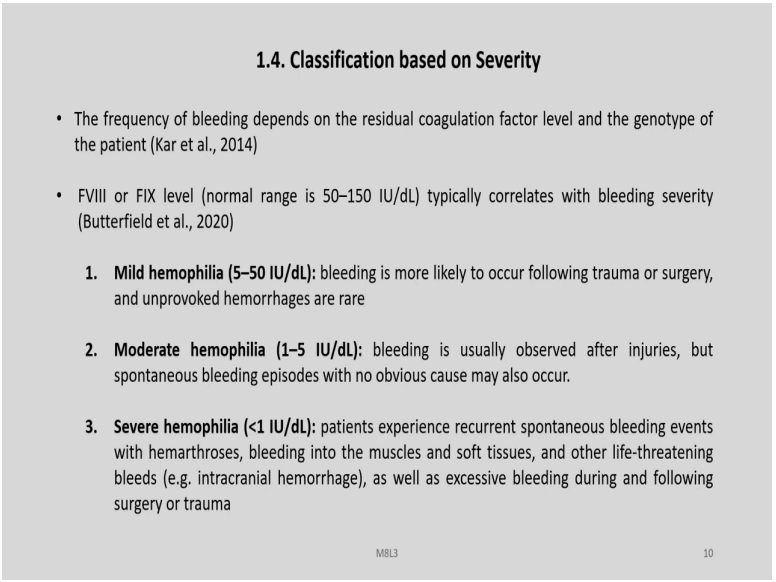

There is a classification of hemophilia based on severity as well. The frequency of bleeding depends on the residual coagulation factor level and the genotype of the patients. Factor VIII or factor IX level a normal range is 50 to 150 IU per dL typically correlates with bleeding severity. So, based on the severity they can be divided into mild, moderate and severe hemophilia. In mild hemophilia, the 5 to 50 IU per dL bleeding is more likely to occur following trauma or surgery and unprovoked hemorrhages are rare.

While in moderate hemophilia, the range is 1 to 5 IU per dL. Bleaning is really observed after injuries, but spontaneous bleeding episodes with no obvious causes may also occur. In severe hemophilia, less than 1 IU per dL patients experience recurrent spontaneous bleeding events with hemarthroses, bleeding into the muscles and soft tissues and other life threatening bleeds as well as excessive bleeding during and following surgery or trauma.

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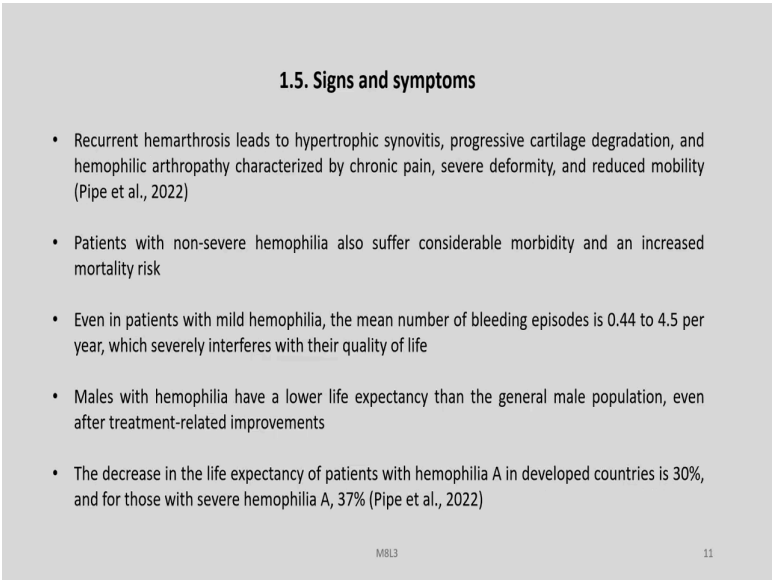

What are the Signs and symptoms of hemophilia? Recurrent hemarthrosis leads to hypertrophic synovitis, progressive cartilage degradation and hemophilic arthropathy characterized by chronic pain, severe deformity and reduced morbidity. Patients with non severe hemophilia also suffers considerable morbidity and an increased mortality risk. Even in patients with mild hemophilia, the mean number of bleeding episodes is 0.44 to 4.5 per year which severely interferes with the quality of life.

Males with hemophilia have a lower life expectancy than the general male population even after treatment related improvements. The decrease in the life expectancy of patients with hemophilia A in developed countries is 30 percent and for those with severe hemophilia A, 37 percent.

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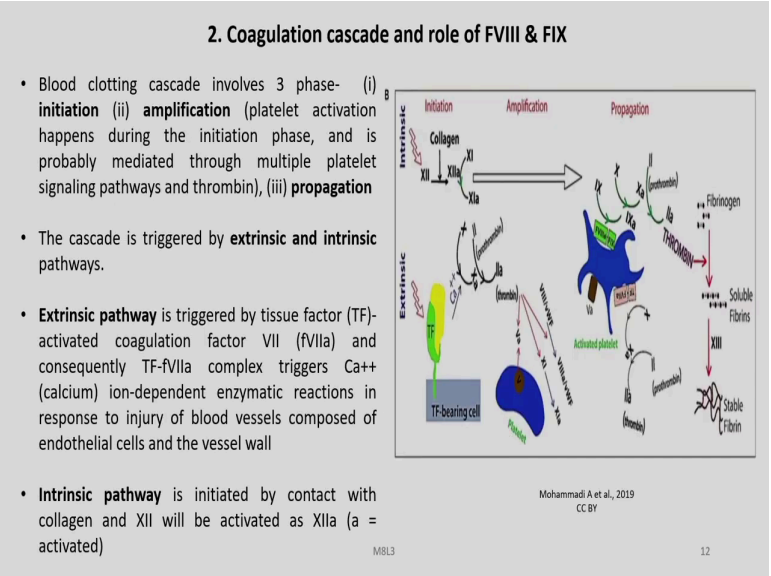

Coagulation cascade and role of factor VIII and factor IX. Blood clotting cascade involves 3 phase initiation, amplification and propagation. The cascade is triggered by extrinsic and intrinsic pathways. Extrinsic pathway is triggered by a tissue factor TF activated coagulation factor VII (fVIIa) and consequently TF fVIIa complex triggers calcium ion dependent enzymatic reactions in response to injury of blood vessels, composed of endothelial cells and the vessel wall.

Intrinsic pathway is initiated by contact with collagen and XII factor will be activated as XIIa or XII activated.

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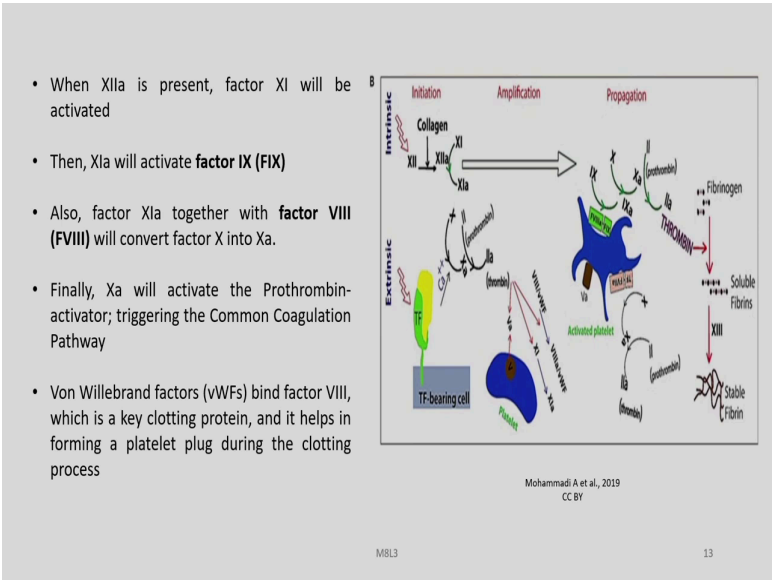

When XII a is present, factor XI will be activated as you can see here in this picture. Then XIa will activate factor IX, also factor XIa together with factor VIII will convert factor X into Xa. Finally, Xa will activate the prothrombin activator triggering the common coagulation pathway.

As you can see over here, Von Willebrand factors bind factor VIII, which is a key clotting protein and it helps in forming a platelet plug during the clotting process.

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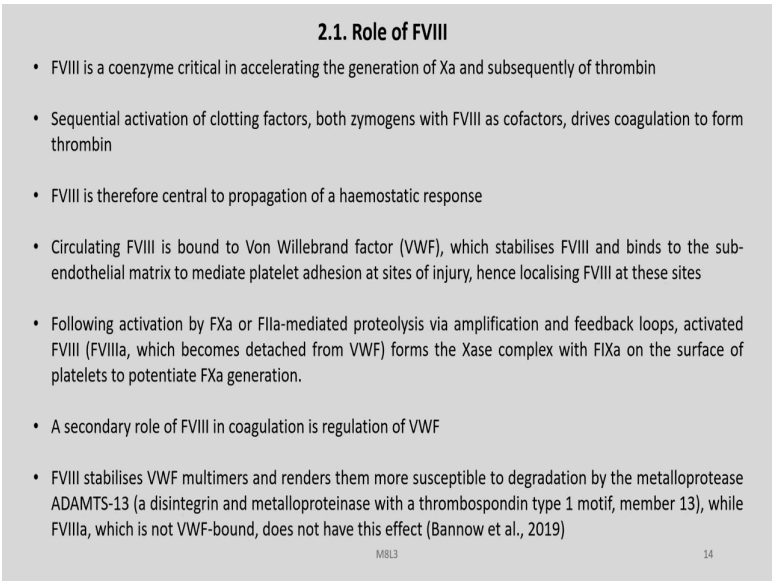

Let us examine the role of factor VIII in little bit of more detail. Factor VIII is a coenzyme critical in accelerating the generation of Xa and subsequently of thrombin. Sequential activation of clotting factors, both zymogens with factor VIII as cofactors, drives coagulation to form thrombin.

Factor VIII is therefore, central to propagation of a haemostatic response. Circulating factor VIII is bound to VWF factor which stabilizes factor VIII and binds to the sub-endothelial matrix to mediate platelet adhesion at sites of injury, hence a localizing factor VIII at these sites. Following activation by factor X a or factor II a mediated proteolysis via amplification and feedback loops, activated factor VIII (factor VIIIa) which becomes detached from VWF forms the Xase complex with factor IXa on the surface of platelets to potentiate factor X generation.

A secondary role of factor VIII in coagulation is regulation of VWF. Factor VIII stabilizes VWF multimers and renders them more susceptible to degradation by the metalloprotease ADAMTS-13, a disintegrin and metalloproteinase is with a thrombospondin type one motif member 13, while factor of VIIIa which is not VWF bound does not have these effect.

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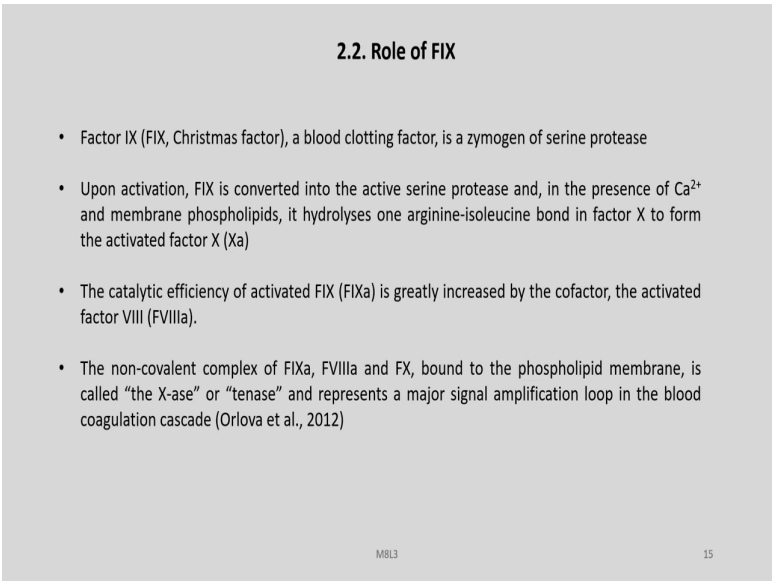

What is the role of factor IX? Factor IX also known as a Christmas factor, is a blood clotting factor, is a zymogen of serine protease. Upon activation factor IX is converted into the active serine protease and in the presence of calcium ions and membrane phospholipids, it hydrolyzes one arginine isoleucine bond in factor X to form the activated factor Xa.

The catalytic efficiency of activated factor IX is greatly increased by the cofactor, the activated factor VIII. The non-covalent complex of factor IXa, factor VIIIa and factor X bound to the phospholipid membrane is called the Xase or Tenase and represents a major signal amplification loop in the blood coagulation cascade.

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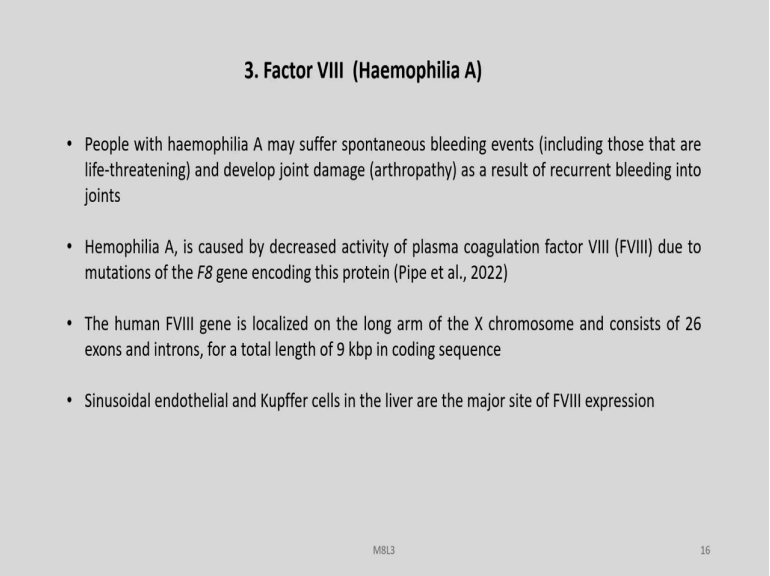

Factor VIII haemophilia A. People with haemophilia A may suffer spontaneous bleeding events including those that are live threatening and develop joint damage, arthropathy as a result of recurrent bleeding in the joints. Hemophilia A is caused by decreased activity of plasma coagulation factor VIII due to mutations of the FVIII gene encoding the protein. The human factor VIII gene sometimes also called HF VIII is localized on the long arm of the X chromosome as we have already known and consists of 26 exons and introns.

For a total length of 9 kilo base pairs in the coding sequence sinusoidal endothelial and kupffer cells in the liver are major sites of factor VIII expression. Let us discuss about the structure of factor VIII protein which is involved in the hemophilia A. The gene encodes a large precursor glycoprotein of 2332 amino acid residues which is the maximum length, consisting of 6 structural domains and 3 acidic sub-domains, organized in a heavy chain -

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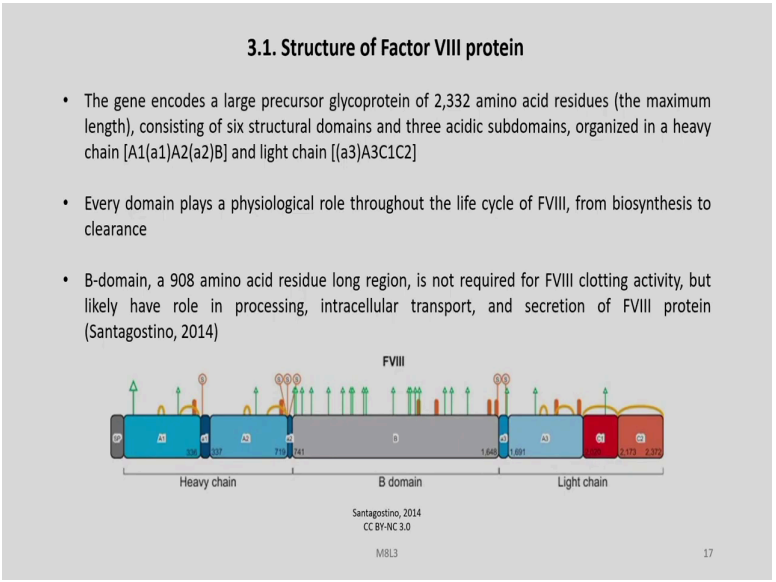

[A1(a1)A2(a2)B] and light chain [(a3)A3C1C2]. Every domain plays a physiological role throughout the life cycle of factor VIII from biosynthesis to clearance. B domain is a 908 amino acid residue long region. It is not required for factor VIII clotting activity, but likely to have a role in processing intracellular transport and secretion of factor VIII protein.

So, you can see here the heavy chains with A a A2 a a2. This is a1 A a3 A3 a C1 B2 and sorry C2 and the B domain.

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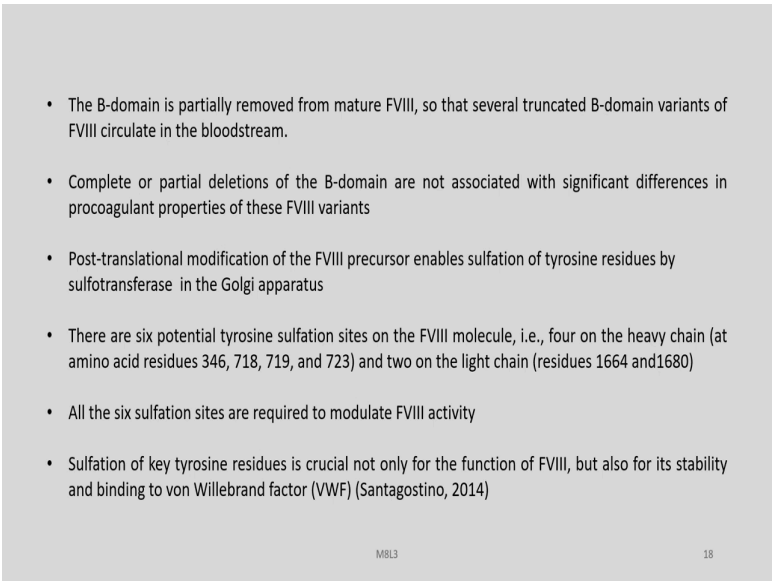

The B domain is partially removed from mature factor VIII, so that several truncated B domain variants of factor VIII circulate in the bloodstream. Complete or partial deletions of the B domain are not associated with significant differences in procoagulant properties of these factor VIII variants. Post translational modification of the factor VIII precursor enables sulfation or tyrosine residues by sulfotransferase in the Golgi apparatus.

There are 6 potential tyrosine sulfation sites on the factor VIII molecule that is 4 on the heavy chain at amino acid residues 346, 718, 719 and 723 and 2 on the light scene residues 1664 and 1680, all the 6 sulfation sites are required to modulate factor VIII activity. Sulfation of key tyrosine residues is crucial not only for the function of factor eight, but also for its stability and binding to Von Willebrand factor.

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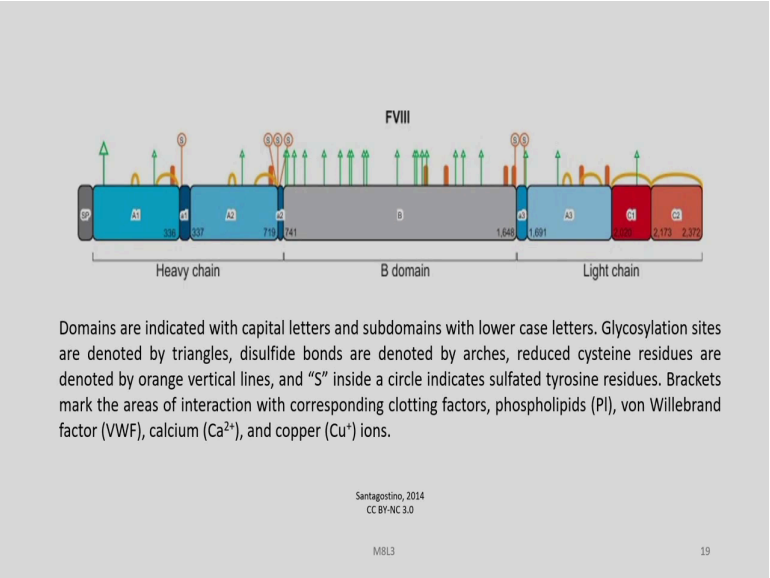

Here, the domains are integrated with capital letters and sub-domains with lowercase letter which I have already explained to you earlier. The glycosylation sites are denoted by the triangles, disulfide bonds are denoted by arches, reduced cysteine residues are denoted by orange vertical lines.

And "S" inside a circle indicates sulfated tyrosine residues. Brackets mark the areas of intercalation with corresponding clotting factors, phospholipids (PI), von Willebrand factor (VWF), calcium ions and copper ions. Let us look into the gene structure of FVIII which produces these factor VIII protein which we have just discussed.

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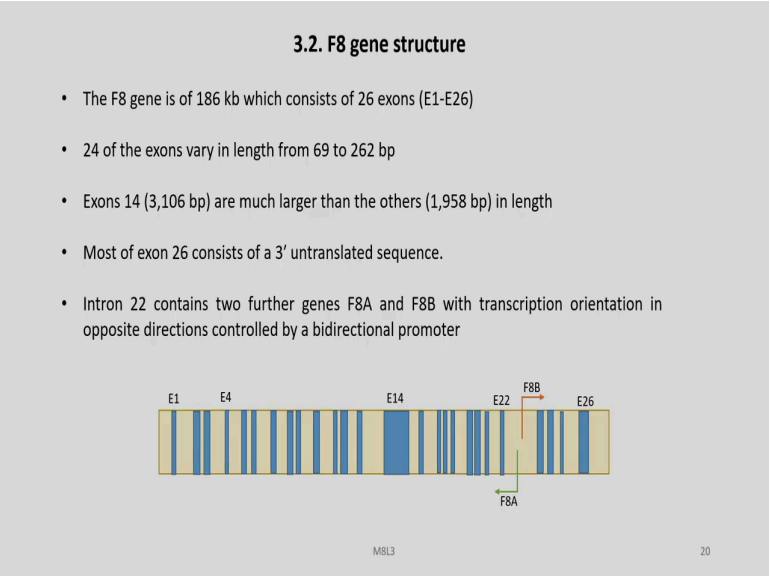

The FVIII gene is of 186 kilo bases which consists of around 26 exons, 24 of the exons vary in length from 69 to 262 base pairs. Exons 14 are much larger than the others in length. Most of the exons 26 consists of the 3 prime untranslated sequences. Intron 22 contains 2 further genes F8A and F8B with transcription orientation in opposite directions controlled by a bidirectional promoter.

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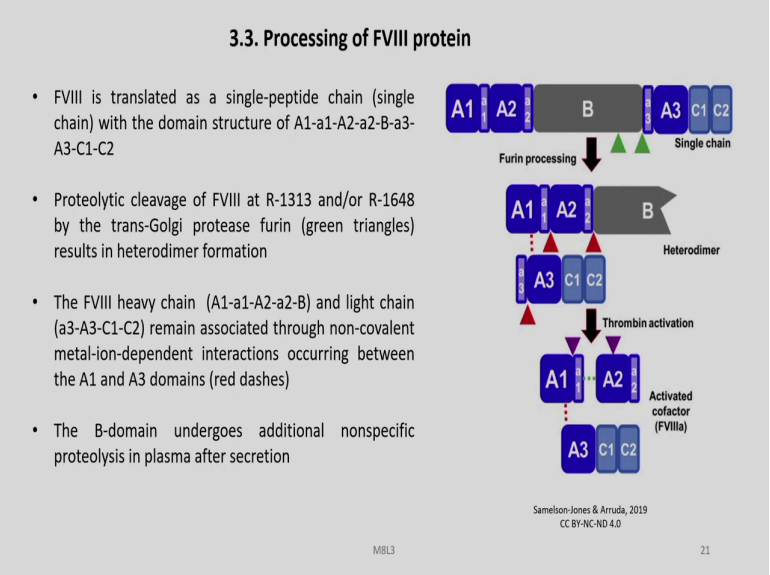

Factor VIII is translated as a single peptide chain with the domain structure of A1, a1, A2, a2, B, a3, A3, C1 and C2 as you can see here into a single chain. Proteolytic cleavage of a factor

VIII at a residue R-1313 and R-1648 by the trans-golgi protease furin as shown in this by these green triangles results in heterodimer formation.

The factor VIII heavy chain consisting of A1 to B and light chain consisting of a3 to C2 remain associated through non-covalent metal ion dependent interactions occurring between the A1 and A3 domains shown by the red dashes. The beta domain undergoes additional nonspecific proteolysis in plasma after secretion.

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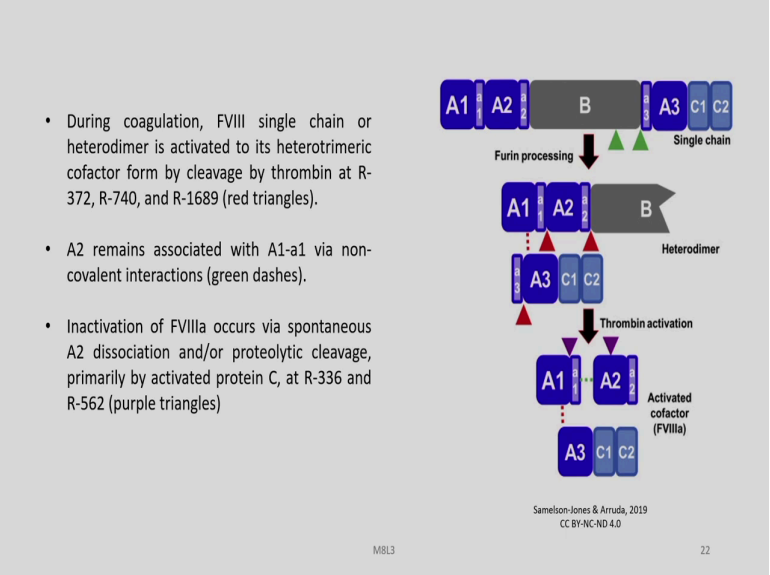

During coagulation, factor VIII single chain or heterodimer is activated to it is heterotrimeric cofactor form by cleavage by thrombin at R372, R740 and R1689 shown by the red triangles. A2 remains associated with A1 and a1 via a non-covalent interactions shown by the green dashes. Inactivation of factor VIIIa occurs via spontaneous A2 dissociation and or proteolytic cleavage primarily by activated protein C at R336 and R562 shown by these purple triangles.

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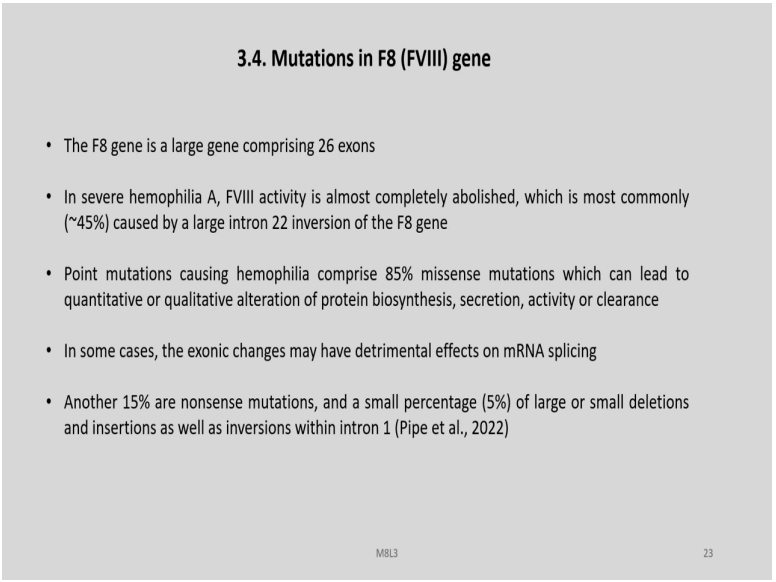

What happens if there are certain mutations in FVIII gene? The FVIII gene is a large gene comprising of 26 exons which we now know. In severe hemophilia A, factor VIII activity is almost completely abolished which is most commonly around 45 percent caused by a large intron to inversion of the FVIII gene.

Point mutations causing hemophilia comprises 85 percent missense mutations, which can lead to quantitative or qualitative alterations of protein biosynthesis, secretion, activity or clearance. In some cases, the exonic changes may have detrimental effects on mRNA splicing. Another 15 percent are nonsense mutations, and a small percentage 5 percent of large or small deletions in insertions as well as inversions within intron 1.

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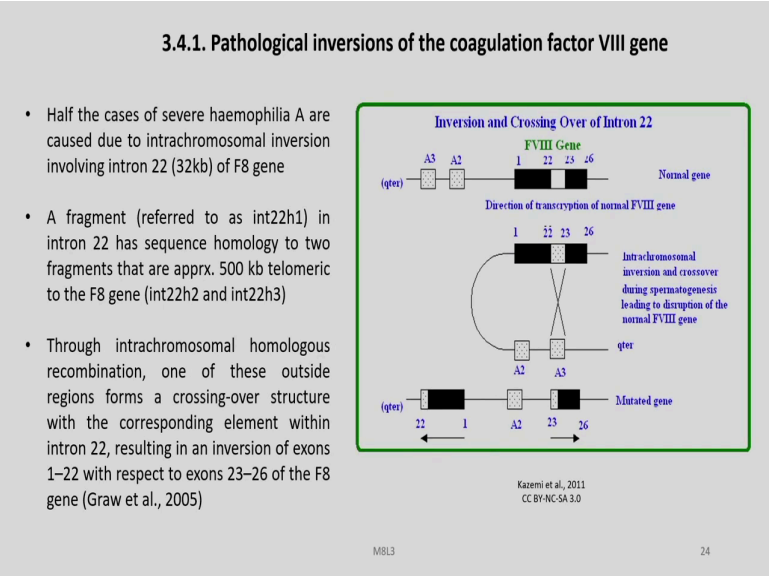

Pathological inversions of the coagulation factor VIII gene. Half the cases of severe haemophilia A are caused due to a intrachromosable inversion involving intron 22 of FVIII gene which we have just discussed. A fragment referred to as int22h1 in intron 22 has sequence homology to two fragments that are approximately 500 kilo base telomeric to the F VIII gene.

Through intrachromosomal homologous recombination, one of these outside regions forms a crossing-over structure with the corresponding element within intron 22, resulting in an inversion of exons 1-22 with respect to exons 23-26 of the FVIII gene. So, here we see the direction of transcription of normal FVIII gene and here we see the intra chromosomal inversion and crossover during spermatogenesis leading to destruction of the normal FVIII gene. And this is the result of the mutated gene as shown in this diagram.

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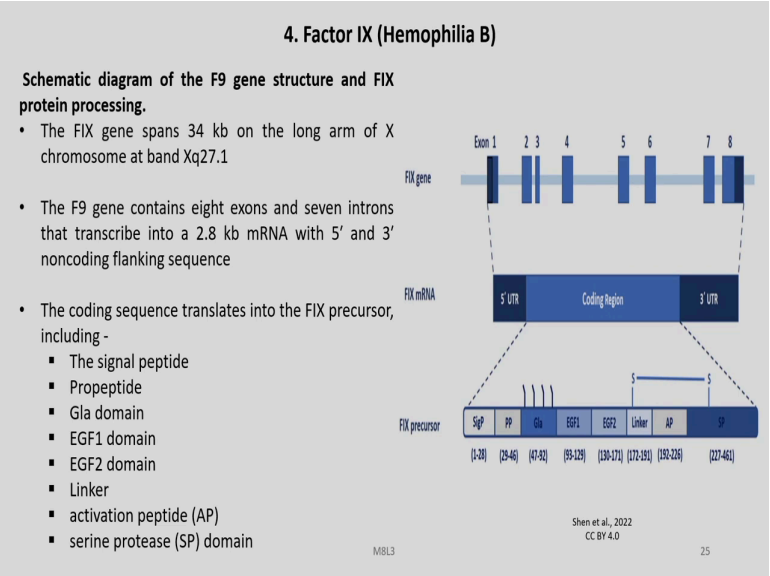

Factor IX hemophilia B. The scematic diagram for FIX gene structure and FIX protein processing is shown in this figure. FIX gene spans 34 kilo bases on the long arm of X chromosome at band Xq27.1. The FIX gene contains 8 exons and 7 introns that transcribe into a 2.8 kilo base mRNA with 5 prime and 3 prime noncoding flanking sequences.

The coding sequence translates into the FIX precursor, including the signal peptide, the pro peptide, the Gla domain, the EGF1 domain, EGF 2 domain, linker, activation peptide and serine protease domain as shown in this figure. This is the FIX precursor.

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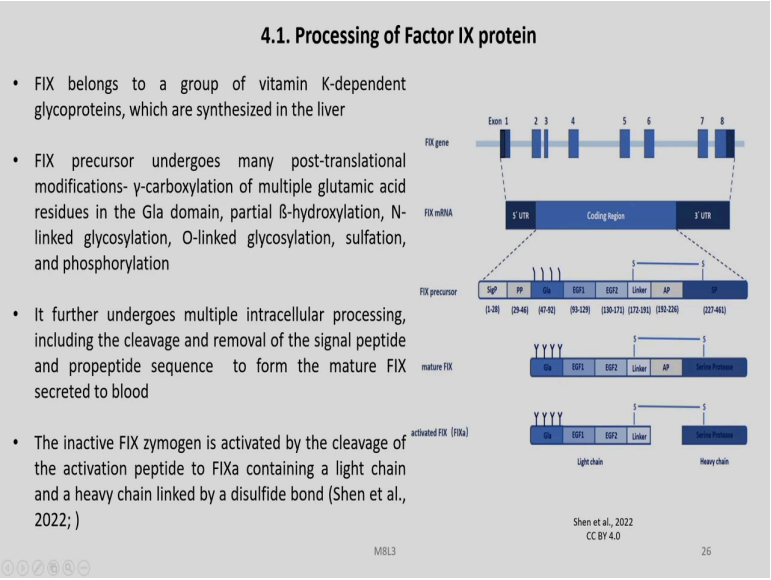

Processing of the factor IX. Protein FIX belongs to a group of vitamin K defendant glycoproteins which are synthesized in the liver.

FIX precursors undergoes many post translational modifications gamma carboxylation of multiple glutamic acid residues in the Gla domain, partial beta hydroxylation and link glycosylation, O-linked glycosylation, sulfation and phosphorylation. It further undergoes multiple intracellular processing including the cleavage and removal of the signal peptide and propeptide, sequence to form the major FIX secreted to blood.

The inactive FIX zymogen is activated by the cleavage of activation peptide to FIXa, containing a light chain and heavy chain linked by disulfate bond as shown in this diagram.

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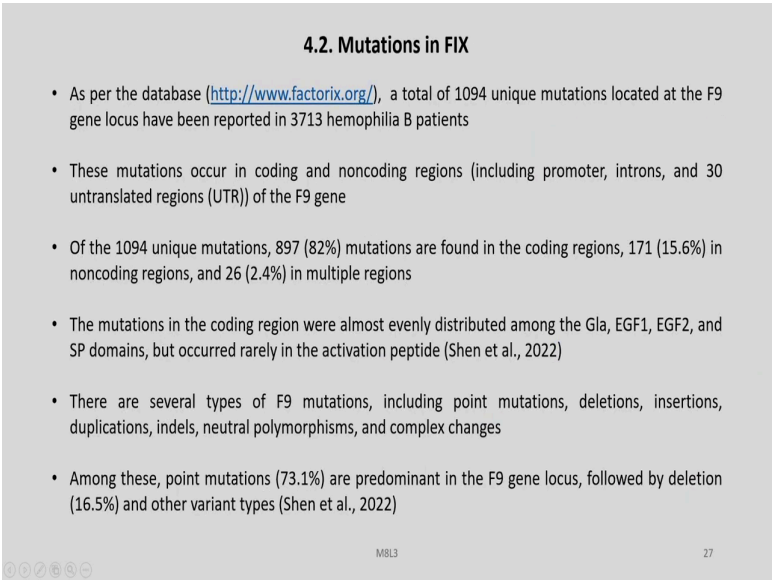

What happens due to certain mutations occurring in FIX factor IX? If you visit this database factorix.org, a total of 1094 unique mutations located at the FIX gene locus have been reported in 3713 hemophilia B patients.

These mutations occur in coding and noncoding regions including promoters, introns and 30 untranslated regions of the FIX gene. Of the 1094 unique mutations, 897 or 82 percent mutations are found in the coding regions, 15.6 percent in non-coding regions and 26 percent in multiple regions. The mutations in the coding regions were almost evenly distributed among the Gla, EGF 1, 2 and SP domains that occurred really in the activation peptide.

There are several types of FIX mutations including point mutations, deletions insertions, duplications, indels, neutral polymorphisms and complex changes. Among these point mutations- 73.1 percent are predominant in the FIX gene locus followed by deletion- 18.5 percent and other variant types. About 88 percent of patients carry point mutations and in only about 12 percent have deletions insertions duplications or indels.

Most deletions, insertions, duplications and indels in a coding sequence cause a frame shift generating a truncated or an extended polypeptide with a changed sequence.

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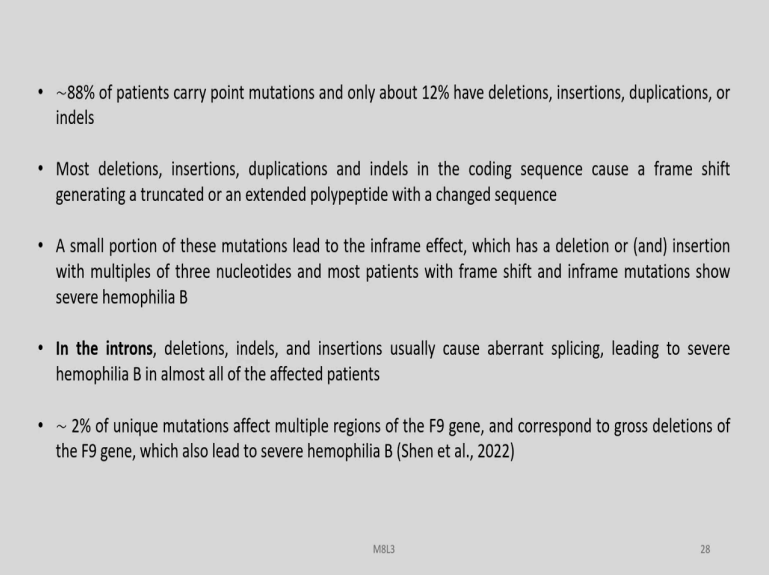

A small portion of these mutations lead to the inframe effect, which is a deletion and or insertion with multiples of 3 nucleotides and most patients with frame shift and in frame mutation show severe hemophilia B. In the introns, deletions, indels and insertions usually cause aberrant splicing leading to severe hemophilia B in almost all of the affected patients.

About 2 percent of unique mutations affect multiple regions of the FIX gene and correspond to gross deletions of the gene which also leads to severe hemophilia B.

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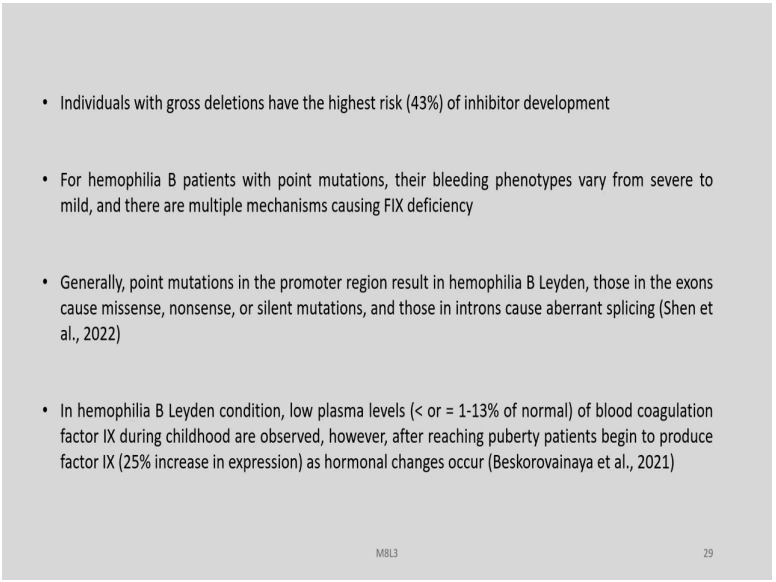

Individuals with gross deletions have the highest risk, about 43 percent of inhibitor development. For hemophilia B patients with point mutations, their bleeding phenotypes vary from severe to mild and there are multiple mechanisms causing factor IX deficiency.

Generally, point mutations in the promoter region result in hemophilia B Leyden, those in the exons cause missense, nonsense or silent mutations and those in the introns cause aberrant splicing.

In hemophilia B Leyden condition, low plasma levels about 1 to 13 of normal of blood coagulation factor IX during childhood are observed. However, after reaching puberty patients begin to produce factor IX, 25 percent increase in expression as the hormonal changes occur. With this we come to end of part A of this lecture, we will be continuing this lecture in part B thank you for your patient hearing.