Genome Editing and Engineering
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Module - 08
Applications of genome editing in treating human diseases
Lecture - 33
Human cell engineering in diseases: Hemophilia - Part B

Welcome to my course on Genome Editing and Engineering. We are discussing about Applications of Genome Editing in treating human diseases. In the earlier lecture, we discussed about Hemophilia we are continuing this lecture as Part – B. What are the available conventional treatments in Hemophilia?

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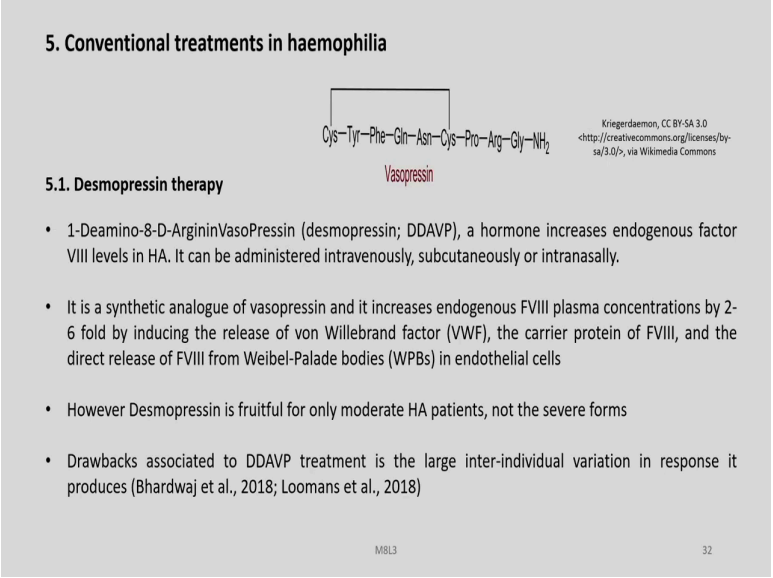

There is a treatment called Desmopressin therapy and before that we have a look at this molecule called Vasopressin about which we will discuss. 1-Deamino-8-D-ArgininVasoPressin also called as desmopressin; DDAVP. It is a hormone increases endogenous Factor 8 levels in hemophilia A. It can be administered intravenously, subcutaneously or intranasally.

It is a synthetic analogue of vasopressin and it increases endogenous factor VIII plasma concentrations by 2 to 6 fold by inducing the release of von Willebrand factor, the carrier protein of factor VIII and the direct release of Factor 8 from Weibel-Palade bodies in

endothelial cells. However, desmopressin is fruitful only for moderate hemophilia A patients and not for those having severe form of the disease. There are several drawbacks associated to DDAVP treatment, particularly large inter individual variation in response, says it produces against its administration.

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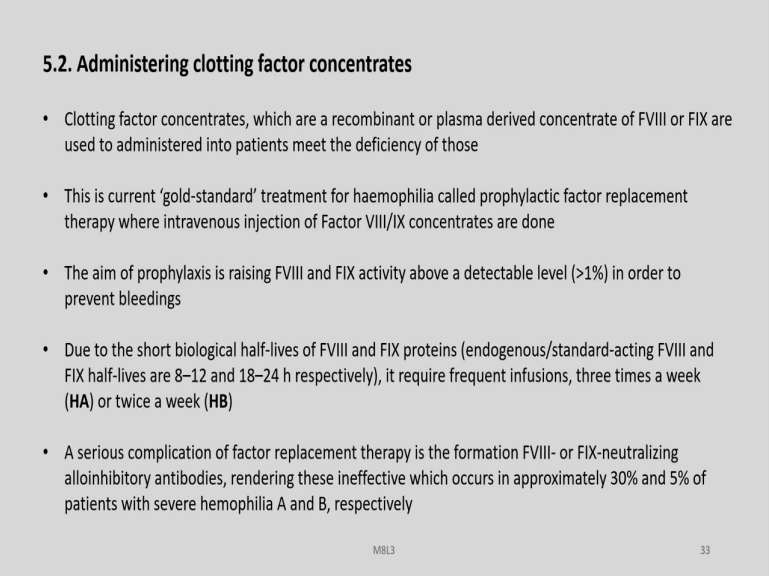

Another conventional therapy is administration of clotting factor concentrates. Clotting factor concentrates which are a recombinant or plasma derived concentrate of factor VIII or IX are used to administer into patients to meet the deficiency of these factors. This is currently the 'gold – standard' treatment for haemophilia, called prophylactic factor replacement therapy where intravenous injection of factor VIII or IX concentrates are given.

The aim of prophylaxis is raising factor VIII and factor IX activity above a detectable level in order to prevent bleedings. Due to the short biological half-lives of factor VIII and factor IX proteins, it requires frequent infusions, three times a week for a HA or twice a week for HB. A serious complication of factor replacement therapy is the formation of factor VIII or factor IX neutralizing alloinhibitory auto antibodies, rendering these ineffective which occurs in approximately 30 percent and 5 percent of patients with severe hemophilia A and B respectively.

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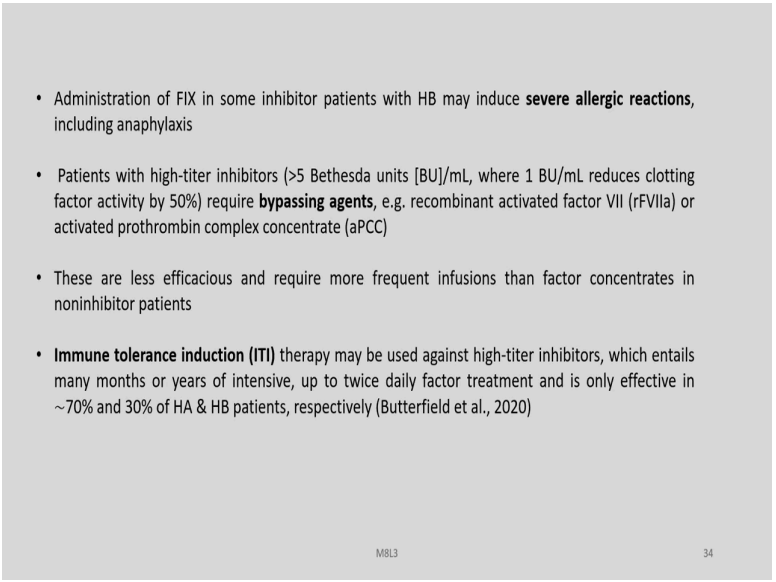

Administration of factor IX in some inhibitor patients with HB may induce severe allergic reactions, including anaphylaxis. Patients with high-titer inhibitors 5 Bethesda unit BU per ml or where 1 Bethesda unit BU per ml reduces clotting factor activity by about 50 percent require bypassing as in example recombinant activated Factor 7 or activated prothrombin complex concentrate.

These are less efficacious and require more frequent infusions than factor concentrates in noninhibitor patients. ITI or immune tolerance induction therapy may be used against high-titer inhibitors, which entails many months or years of intensive, up to twice daily factor treatment and is only effective in 70 percent and 30 percent of HA and HB patients respectively.

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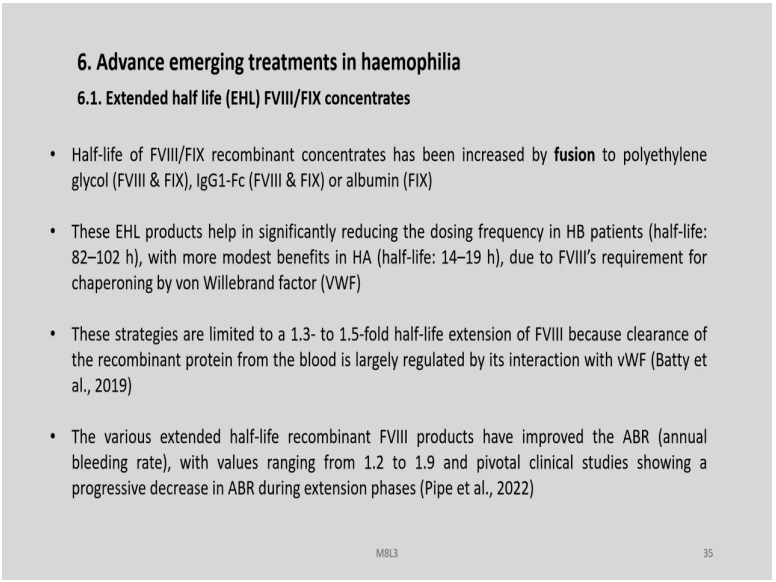

What are the some of the advanced emerging treatments in haemophilia? One is extended half-life of factor VIII and IX concentrates. Half-life of factor VIII, IX recombinant concentrates have been increased by fusion to polyethylene glycol IgG1-Fc or albumin. These extended half-life products help in significantly reducing the dosing frequencies in HB patients with more modest benefits in a hemophilia A where half-life is 14 to 19 hours, due to factor eights requirements for chaperoning by von Willebrand factor.

These strategies are limited to a 1.3 to 1.5 fold half-life extension of Factor VIII because clearance of the recombinant protein from the blood is largely regulated by it is interaction with von Willebrand factor. The various extended half-life recombinant factor VIII products have improved the ABR, annual bleeding rate, with values ranging from 1.2 to 1.9 and pivotal clinical studies showing a progressive decrease in ABR during extension phases.

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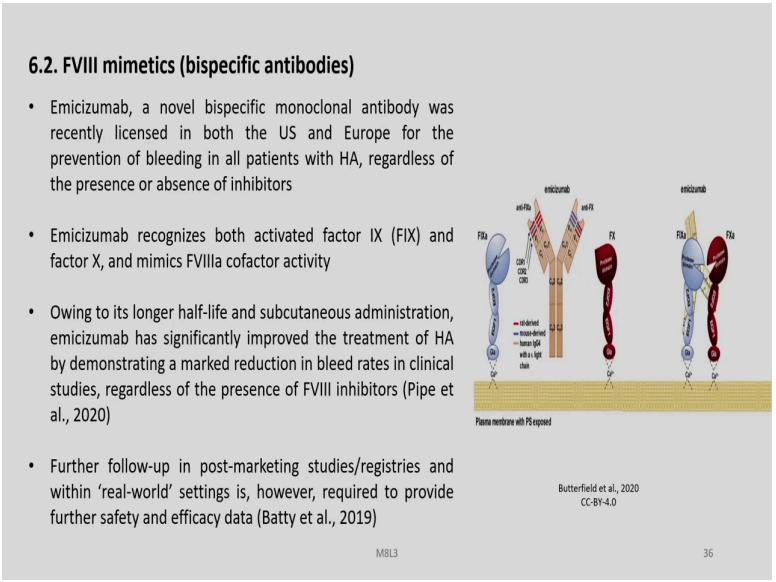

Factor VIII mimetics or bispecific antibodies. Emicizumab, a novel bispecific monoclonal antibody has been licensed in both the US and Europe for the prevention of bleeding in all patients with HA, regardless of the presence or absence of inhibitors. Emicizumab recognizes both activated factor IX and factor X and mimics factor VIIIa cofactor activity.

Owing to its long half-life and subcutaneous administration, Emicizumab has significantly improved the treatment of hemophilia A by demonstrating a marked reduction in bleeding rates in clinical studies, regardless of the presence of factor VIII inhibitors. Further follow-up in post-marketing studies and within 'real world' settings is however, required to provide further safety and efficacy of Emicizumab.

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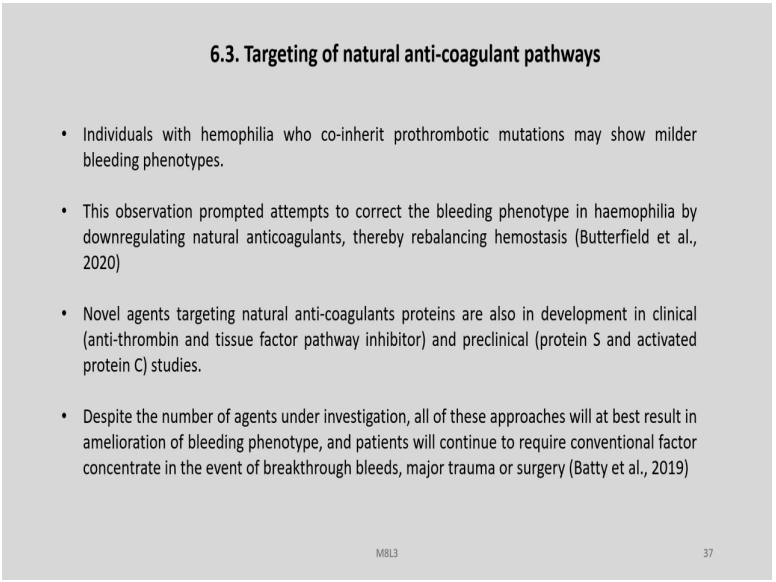

Targeting of natural anti-coagulant pathways. Individuals with hemophilia who co-inherit prothrombotic mutations may show milder bleeding phenotypes. This observation prompted attempts to correct the bleeding phenotypes in hemophilia by down regulating natural anticoagulants, thereby in rebalancing hemostasis. Novel agents targeting natural anti-coagulants proteins are also in development in clinical and pre-clinical studies.

Despite the number of agents under investigation, all of these approaches will at best result in amelioration of bleeding phenotype and patients will continue to require conventional factor concentrate in the event of breakthrough bleeds, major trauma or surgery.

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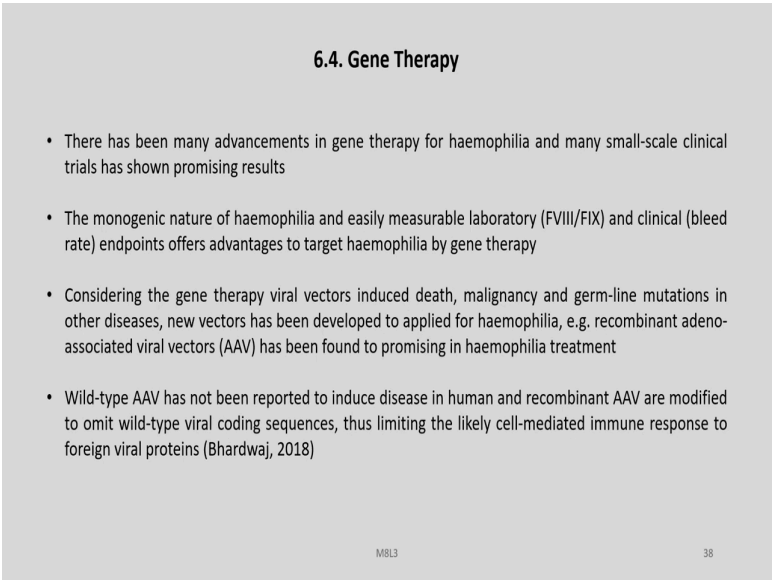

So, to overcome all these disadvantages one of the advancement has been made in the area of gene therapy. There have been many advancements in gene therapy for hemophilia in many small-scale clinical trials has shown promising results. The monogenic nature of haemophilia as already stated and easily measurable laboratory factor VIII, factor IX and clinical bleed rate endpoints offer advantages to target hemophilia by gene therapy.

Considering the gene therapy viral vectors induced death, malignancy and germ-line mutations in other diseases, new vectors have been developed to apply in the case of haemophilia, example recombinant adeno associated viral vectors have been found to be promising in haemophilia treatment. A wild-type AAV has not been reported to induce disease in human and recombinant AAV are modified to omit wild type viral coding sequences, thus limiting likely cell mediated immune responses to foreign viral proteins.

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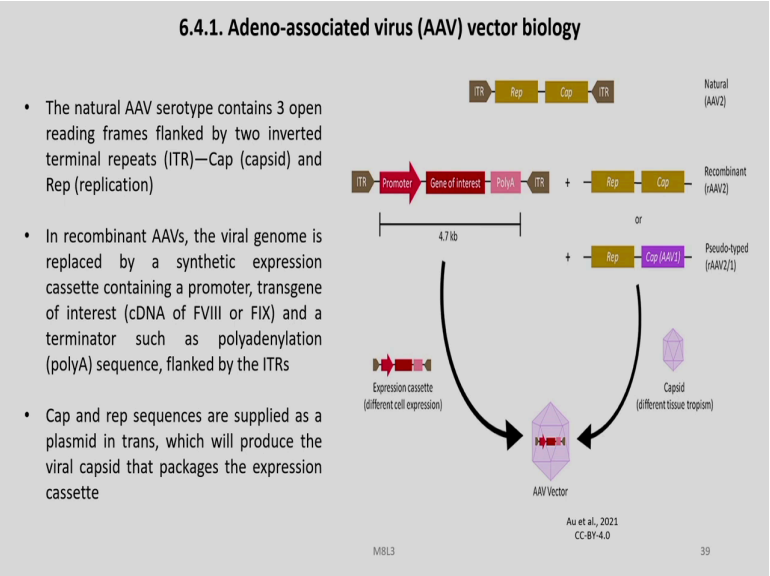

Let us have a small discussion on the adeno-associated virus vector biology, the natural AAV stereo serotype contains 3 open reading frames flanked by two inverted terminal repeats ITR- capsid and replication. In recombinant AAVs, the viral genome is replaced by a synthetic expression cassette containing a promoter, transgene of interest- cDNA of factor VIII or factor IX in this case and a terminator such as a polyadenylation sequence, flanked by the ITRs.

Cap and rep sequences are supplied as a plasmid in trans, which will produce the viral capsid that package is the expression cassette.

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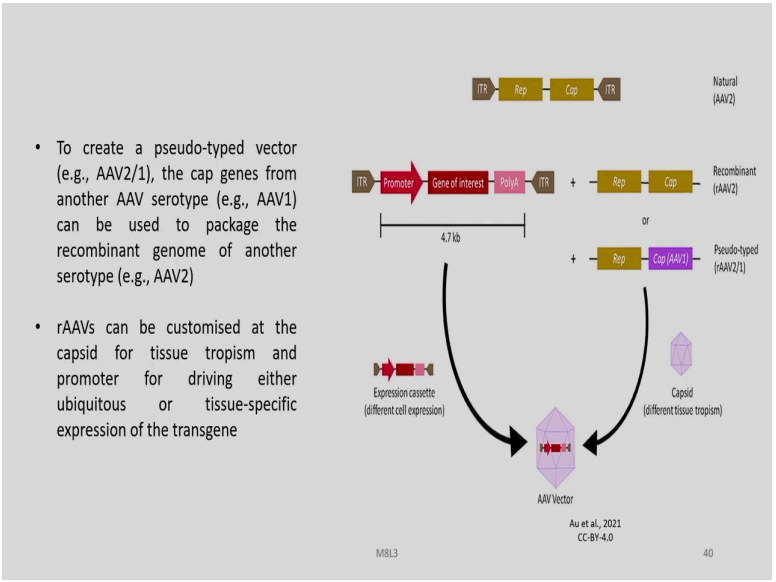

To create a pseudo-type vector example AAV2/1, the cap genes from another AAV serotype can be used to package the recombinant genome of another serotype. rAAVs can be customized at the capsid for tissue tropism and promoter for driving either ubiquitous or tissue - specific expression of the transgene.

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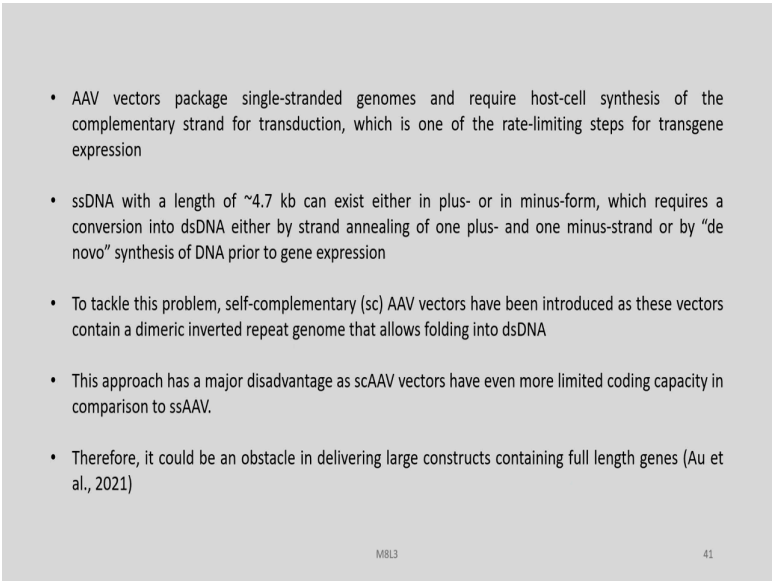

AAV vectors package single stranded genomes and require host cell synthesis of the complementary strand for transduction, which is one of the rate-limiting steps for transgene expression. Single stranded DNA with a length of about 4.7 kilo bases can exist either in plus

or in minus form, which requires a conversion into double stranded DNA either by strand annealing of one plus and one minus strand or by “de novo” synthesis of DNA prior to gene expression.

To tackle this problem, self-complementary sc AAV vectors have been introduced as these vectors contain a dimeric inverted repeat genome that allows folding into double stranded DNA. This approach has a major disadvantage as scAAV vectors have even more limited coding capacity in comparison to scAAV. Therefore, it could be an obstacle in delivering large constructs containing full length genes.

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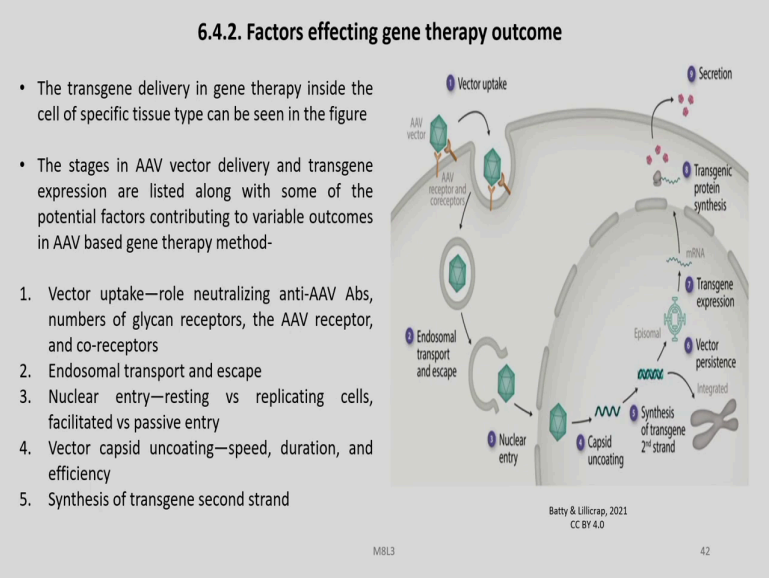

What are the factors that affect the gene therapy outcome? The transgene delivery in gene therapy inside the cell of specific tissue can be seen in this picture. The vector uptake is happening over here and there is endosomal transport and escape and nuclear entry after which there is capsid uncoating, synthesis of transgene then there is vector persistence transient expression and transgenic protein synthesis and finally, secretion taking place.

The stages in AAV vector delivery and transient expression are listed along with some of the potential factors contributing to variable outcomes in AAV based gene therapy method. We will discuss all these steps briefly as follows. Vector uptake- role neutralizing anti-AAV Abs, numbers of glycan receptors, the AAV receptor and co receptors are very important. Endosomal transport and escape following this vector uptake and then the nuclear entry and

uncoating the synthesis of transient of the second strand and finally, expression of the transient and protein synthesis in secretion.

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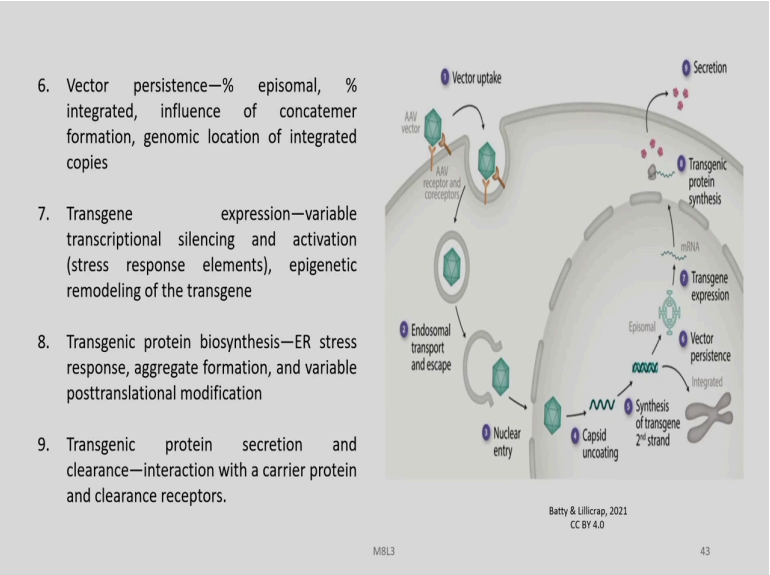

The vector persistence percentage episomal percentage integrated influence of concatemer formation, genomic location of integrated copies all play important roles. The transgene expression - variable transcriptional silencing and activation particularly with stress response elements epigenetic remodeling of the transgene are also equally decisive.

Transgenic protein synthesis, the ER stress responses, aggregate formation and variable posttranslational modification plays important roles. The in the secretion of the proteins and clearance interaction with the carrier protein and clearance receptors are also very very crucial.

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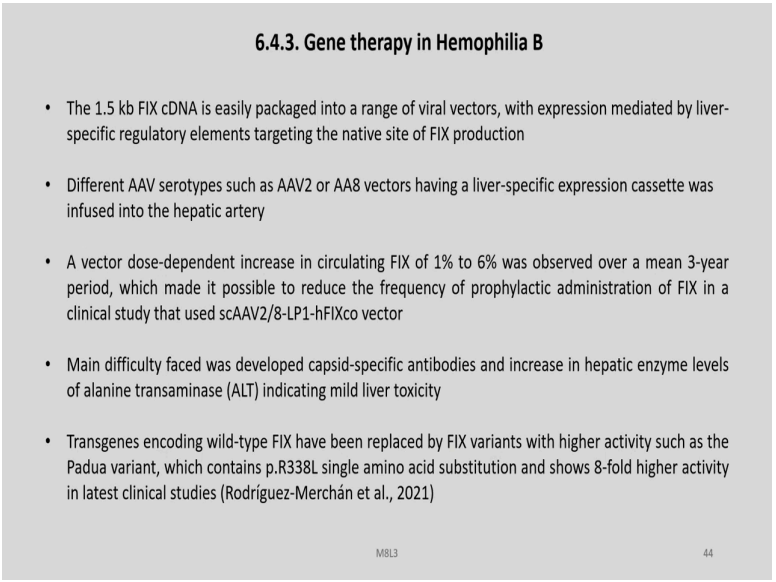

Gene therapy in hemophilia B. The 1.5 kb cDNA is easily packaged into a range of viral vectors, with expression mediated by liver specific regulatory elements targeting the native site of FIX production. Different AAV stereotypes such as AAV2 or AAV8 vectors having a liver - specific expression cassette was infused into the hepatic artery.

A vector dose-dependent increase in circulating FIX or 1 percent to 6 percent was observed over a mean 3 year period, which made it possible to reduce the frequency of prophylactic administration of factor IX in a clinical study that used sc AAV2/8-LP-1-hFIXco vector. Main difficulty faced in this case was developed capsid specific antibodies and increasing hepatic enzyme levels of alanine transaminase indicating mild liver toxicity.

Transgenes encoding wild-type factor IX have been replaced by factor IX variants with higher activity such as the Padua variant, which contains a p.R338L single amino acid substitution and shows 8 fold higher activity in latest clinical studies.

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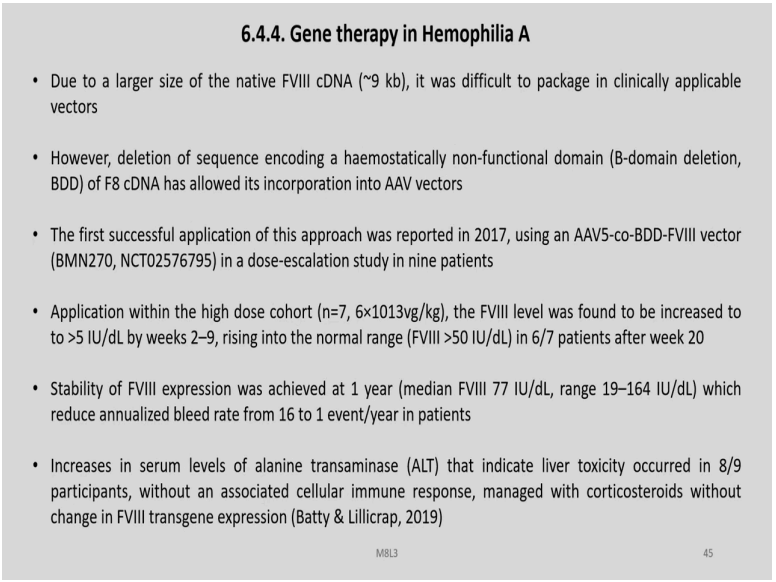

Gene therapy in hemophilia A. Due to a larger size of the native factor VIII cDNA which is about 9 kb, it was difficult to package in clinically applicable vectors. However, deletion of sequence encoding a haemostatically statistically sorry I will repeat (Refer Time: 15:03) ok. Gene therapy in hemophilia A. Due to a larger size of the native factor VIII cDNA which is about 9 kb, it was difficult to package in clinically applicable vectors.

However, deletion of sequence encoding a haemostatically non-functional domain- B-domain deletion of factor VIII cDNA has allowed its incorporation into AAV vectors. The first successful application of this approach was reported in 2017 using an AAV5-co-BDD factor VIII vector in a dose-escalation study in 9 patients. Application within the high dose cohort, the factor VIII level was found to be increased to more than 5 IU by dL by weeks 2 to 9 rising into the normal range in 6 to 7 patients after week 20.

Stability of factor VIII expression was achieved at 1 year, which reduced and annualized bleed rate from 16 to 1 event per year in patients and it is a remarkable achievement. Increases in serum levels of alanine transaminase that indicate liver toxicity occurred in 8 out of 9 participants, without an associated cellular immune response, managed with corticosteroids without change in factor VIII transgene expression.

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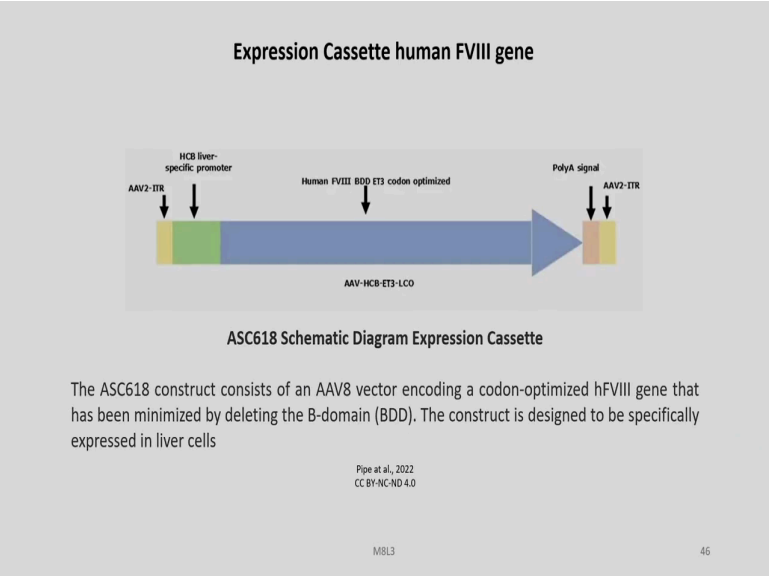

Let us look into the expression cassette of human factor VIII gene. This is the schematic diagram of the expression cassette. The ASC618 construct consists of an AAV 8 vector encoding a codon-optimized human factor VIII gene that has been minimized by deleting the beta domain which has no any function. The construct is designed to be specifically expressed in liver cells.

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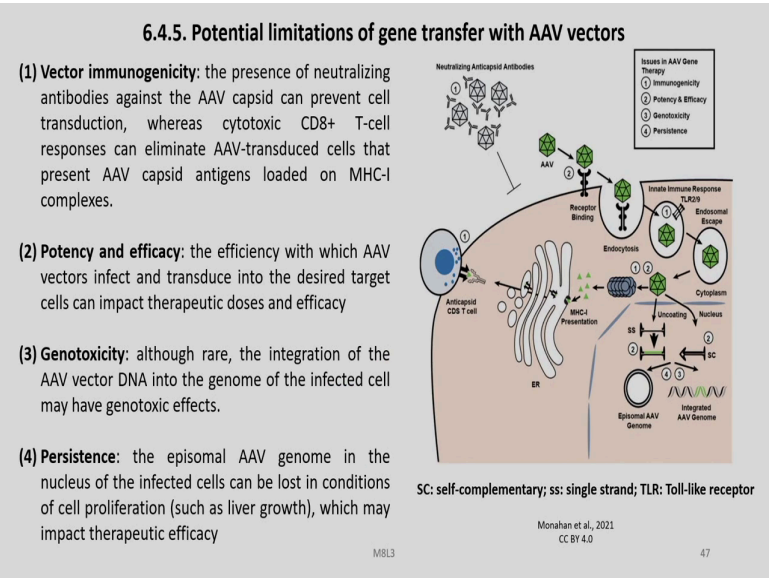

However, there are certain potential limitations of gene transfer with AAV vectors like vector immunogenicity, the presence of neutralizing antibodies against the AAV capsid can prevent

cell transaction whereas, cytotoxic CD 8 plus T cell responses can eliminate AAV-transduced cells that present AAV capsid antigens loaded on MHC-1 complexes. And the second is a potency and efficacy, the efficiency with which AAV vectors infect and transduce into the desired target cells can impact therapeutic doses and efficacy.

And third is the genotoxicity, although rare, the integration of the AAV vector DNA into the genome of the infected cell may have genotoxic effects. Fourth is persistence the episomal AAV genome in the nucleus of the infected cells can be lost in conditions of cell proliferation such as liver growth, which may impact therapeutic efficacy.

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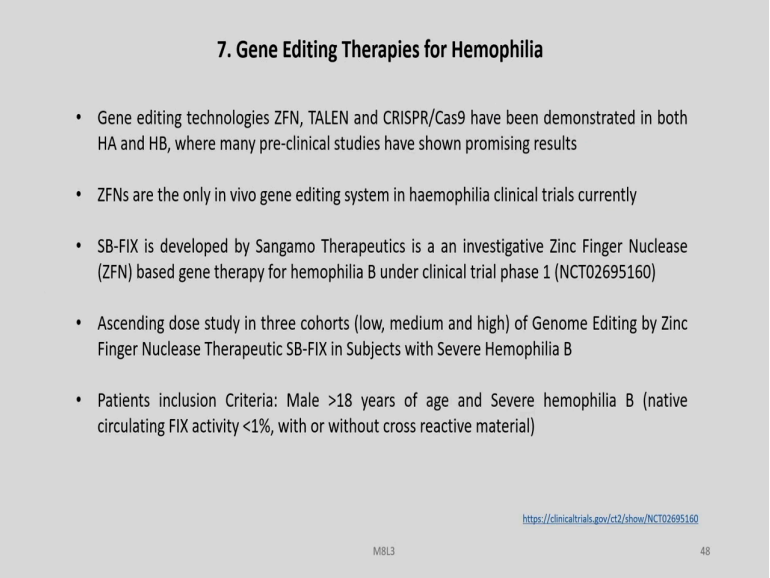

Beyond gene therapy, now there are several developments in the area of gene editing therapies for applications in hemophilia. Gene editing technologies like ZFN, TALEN and CRISPR Cas9 have been demonstrated in both hemophilia A and hemophilia B where many pre-clinical studies have shown promising results. ZFNs are the only in vivo gene editing system in hemophilia clinical trials currently.

SB-factor IX is developed by Sangamo Therapeutics, which is an investigative Zinc Finger Nuclease based gene therapy for hemophilia B under clinical trial phase 1. Ascending dose study in three cohorts- low, medium and high of Genome editing by Zinc Finger Nuclease Therapeutic SB-FIX in subjects with severe hemophilia B has been conducted. Patients inclusion criteria- where males more than 18 years of age and severe hemophilia B having

native circulating factor IX activity more than 1 percent with or without cross reactive material.

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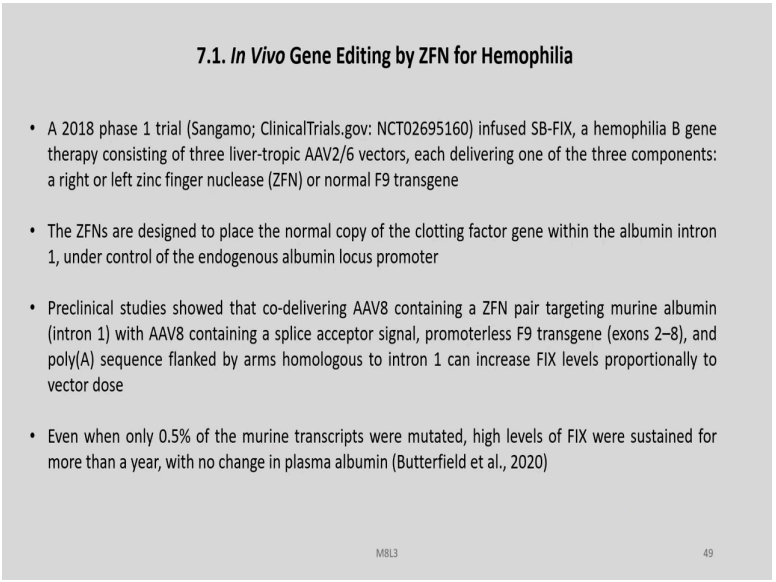

Let us discuss about the in vivo gene editing by ZFN for hemophilia. A 2018 phase 1 trial, infused SB factor IX of hemophilia B gene therapy consisting of three liver-tropic AAV2/6 vectors, each delivering one of the three components: a right or left zinc finger ZFN or a normal FIX transgene. The ZFNs are designed to place the normal copy of the clotting factor gene within the albumin intron 1 under control of the endogenous albumin locus promoter.

Preclinical studies showed that co-delivering AAV8 containing a ZFN pair targeting murine albumin intron 1 with AAV8 containing a splice acceptor signal, promoter less FIX transgene exon 2-8 and poly a sequence flanked by homologous arms to intron 1 can increase FIX levels proportionally to the vector dose. Even when only 0.5 percent of the murine transcripts were mutated, high levels of FIX were sustained for more than a year, with no change in plasma albumin. Ok.

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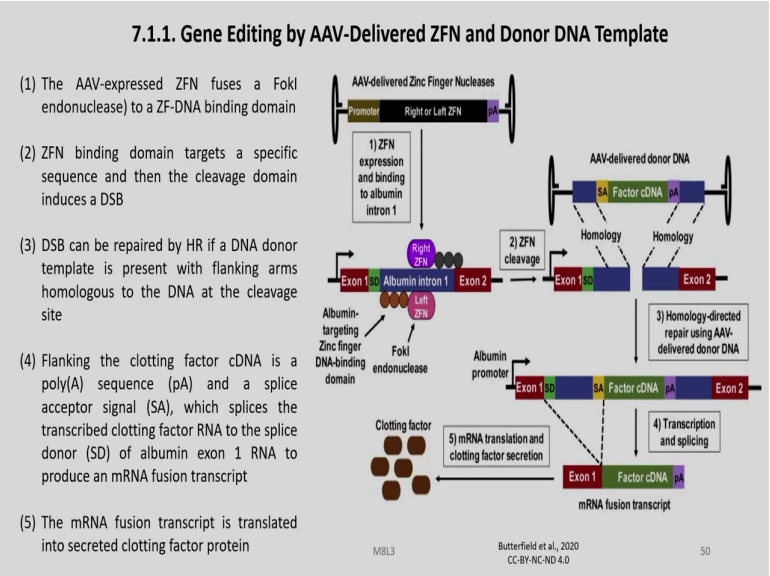

So, let us look into the schematics of gene editing by AAV delivered zinc figure nucleases and donor DNA template. The AAV expressed ZFN fuses a FokI endonuclease to ZF-DNA binding domain as done usually. ZFN binding domain targets a specific sequence and then the cleavage domain induces a double strand break. DSB can be repaired by homologous recombination if a donor DNA template is present with flanking arms homologous to the DNA at the cleavage site.

Flanking the clotting factor cDNA is a polyA sequence pA as you can see here, which splices the transcribed clotting factor RNA to the splice donor SD of albumin exon 1 RNA to produce an mRNA fusion transcript. The mRNA fusion transcript is translated into secreted clotting factor proteins finally.

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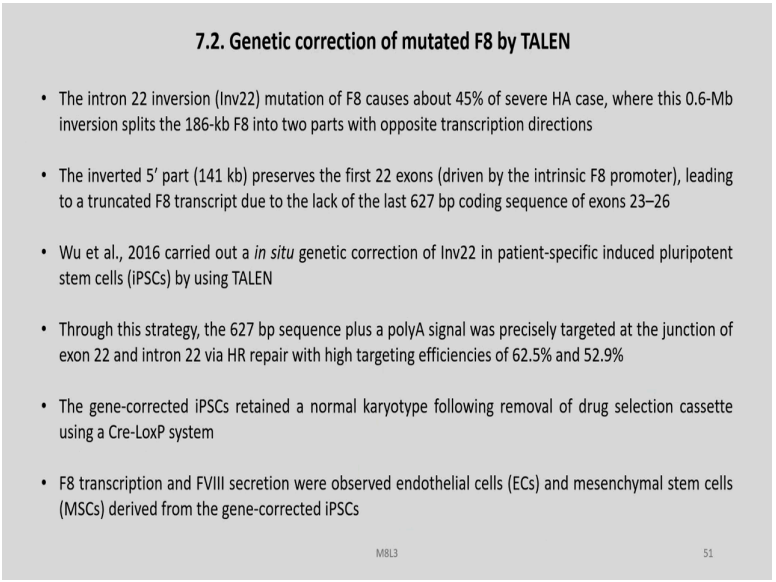

There have been other attempts using TALEN, gene editing technologies for genetic correction of mutated F8 gene. The intron 22 inversion which we have discussed in part A, a mutation of F8 causes about 45 percent of severe hemophilia A case, where this 0.6 Mb inversion splits the 186-kb F8 gene into two parts with opposite transcription directions.

The inverted 5 prime part preserves the first 22 exons, leading to a truncated F8 transcript due to the lack of the last 627 base pair coding sequences of exons 23 to 26. Wu et al, 2016 carried out an in situ generic correction of inversion 22 in patient specific induced pluripotent stem cells by using TALEN. Through this strategy, the 627 base pair sequence plus a polyA signal was precisely targeted at the junction of exon 22 and intron 22 via homologous recombination repair with high targeting efficiencies of 62.5 and 52.9 percent respectively.

The gene corrected iPSCs retained a normal karyotype following removal of drug selection cassette using a Cre-LoxP system. F8 transcription and factor VIII secretion were observed endothelial cells and mesenchymal stem cells derived from the gene connected iPSCs.

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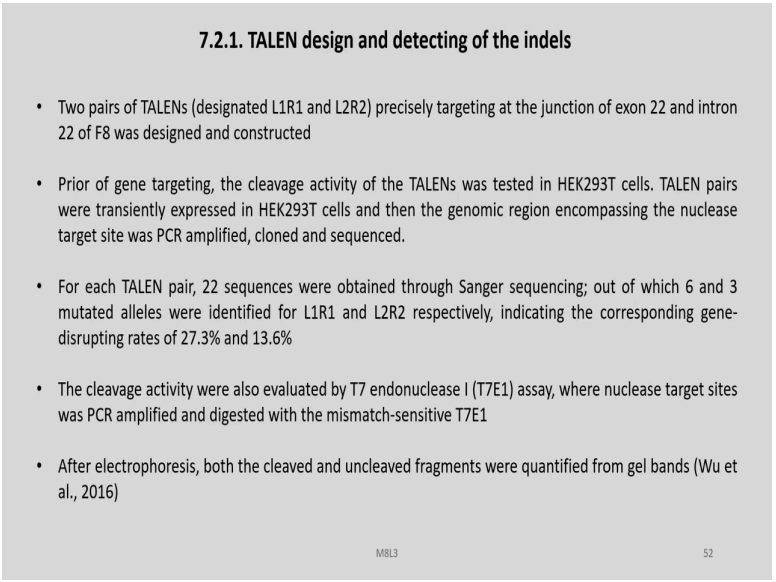

There have been also some effort in TALEN design and detection of the indels, two pairs of the TALENs precisely targeting at the junction of exon 22 and intron 22 of F8 was designed and constructed. They are designed as L1R1 and L2R2. Prior to gene targeting, the cleavage activity of the TALENs were tested in HEK 293 T cells. TALEN pairs were transiently expressed in HEK293T cells and then the genomic regions encompassing the nuclease target site was PCR amplified, cloned and sequenced.

For each TALEN pair, 22 sequences were obtained through Sanger sequencing; out of which 6 and 3 mutated alleles were identified for L1R1 and L2R2 respectively, indicating the corresponding gene disrupting rates of 27.3 percent and 13.6 percent respectively. The cleavage activity were also evaluated by T7 endonuclease assay, where nucleus target sites was PCR amplified and digested with the mismatch sensitive T7E1. After electrophoresis both the cleaved and uncleaved fragments were quantified from gel bands.

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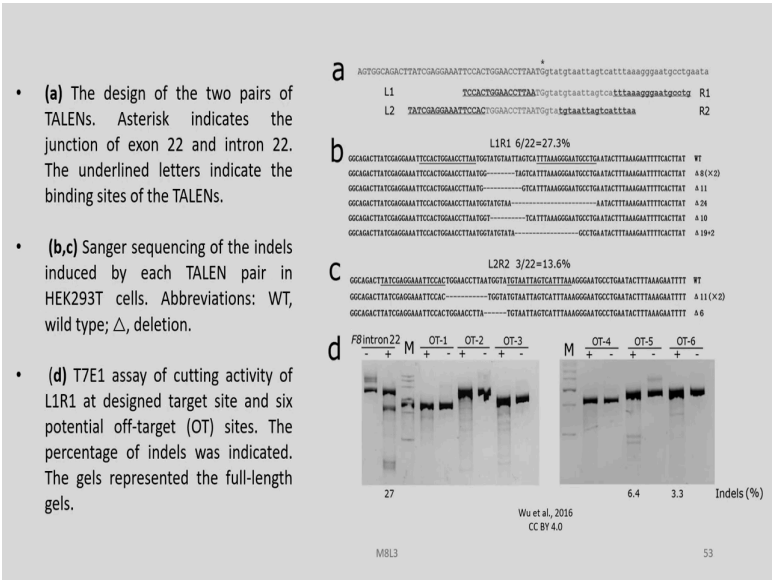

So, here you can see the various results as presented in figure a b c d. 'a' is the design of two pairs of the TALENs L1 and L2. Asterisk indicates the junction of exon 22 and intron 22. The underlined letters indicate the binding sites of the TALENs. In b and c, we can see the results of the Sanger sequencing of the indels induced by its TALEN pair in HEK293T cells, WT, stands for wild-type and the delta is standing for deletion.

In figure d, we can see the T7E1 assay of cutting activity of L1R1 at designed target site and 6 potential off target sites and the percentage of indels was indicated in the figure. The gels represented in the full length gels.

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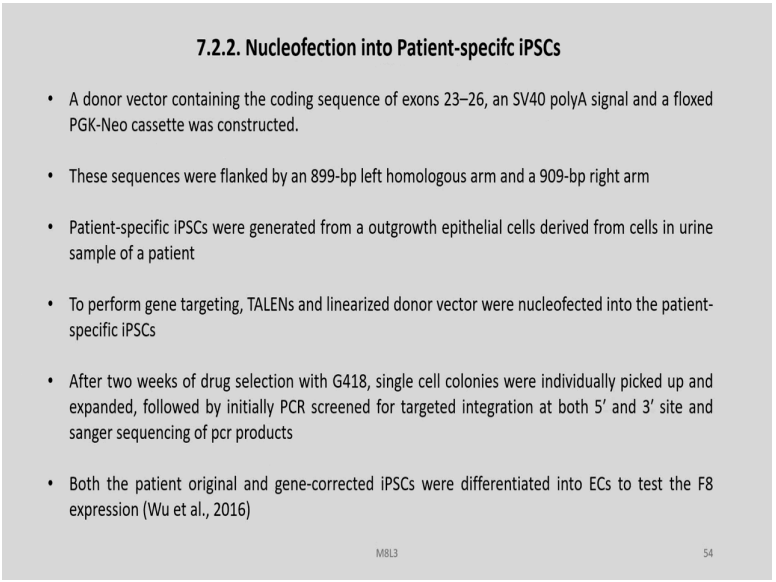

Nucleofection into patient specific iPSCs. A donor vector containing the coding sequence of exon 23-26, an as SV40 poly A signal and a floxed PGK-Neo cassette was constructed. These sequences were flanked by an 899 base pair left homologous arm and a 909 base pair right arm. Patient-specific iPSCs were generated from an outgrowth epithelial cells derived from cells in urine sample of a patient. To perform gene targeting, TALENs and linearized donor vectors were nucleofected into the patients-specific iPSCs.

After two weeks of drug selection with G418, single cell colonies were individually picked up and expanded, followed by initially PCR screened for targeted integration at both 5 prime and 3 prime size and Sanger sequencing of PCR products. Both the patient original and gene corrected iPSCs were differentiated into ECs to test the F 8 expression.

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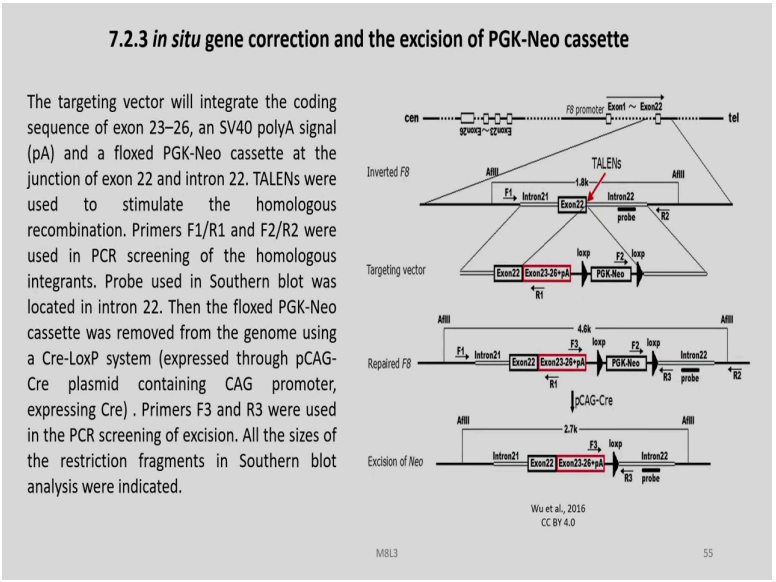

The targeting vector will integrate the coding sequence of exon 23-26 and SV40 polyA signal and a floxed PGK-Neo cassette at the junction of exon 22 and intron 22. TALENs were used to stimulate the homologous recombination. Primers F1/R1 and F2/R2 were used in PCR screening, you can see here F1 and R2 and F2 and R1. Probe used in Southern blot was located in intron 22.

Then the floxed PGK-Neo cassette was removed from the genome using a Cre-loxP system. Primers F3 and R3 were used in the PCR screen of excision. All the sizes of the restriction fragments in Southern blot analysis are also shown.

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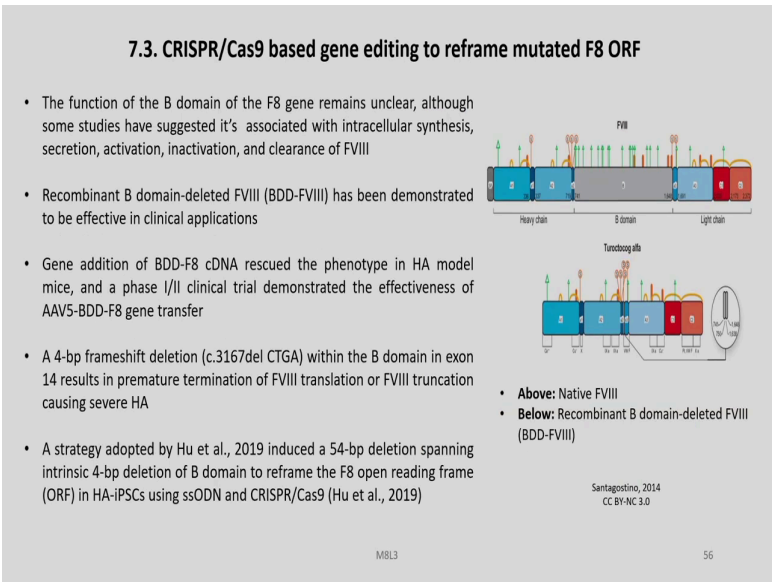

One of the recent approach is the use of CRISPR Cas9 based gene editing to reframe mutated factor VIII open reading frames. The function of the beta domain of the F8 gene remains unclear, although some studies have suggested it is associated intracellular synthesis, secretion, activation, inactivation and clearance of factor VIII while some say it is not very of crucial importance. Recombinant B domain deleted factor VIII BDD minus factor VIII has been demonstrated to be effective in clinical applications.

Gene addition of BDD minus F8 cDNA rescued the phenotype in HA model mice in a phase 1/2 clinical trial demonstrated the effectiveness of AAV5 BDD minus F8 gene transfer. A 4 base pair frameshift deletion within the beta domain B domain in exon 14 results in premature termination of factor VIII translation or factor VIII truncation causing severe HA.

A strategy adopted by Hu et al, 2019 induced a 54 base pair deletion spanning intrinsic 4 base pair deletion of B domain to reframe the F8 open reading frame in HA-induced pluripotent stem cells using single stranded ODN and CRISPR Cas 9.

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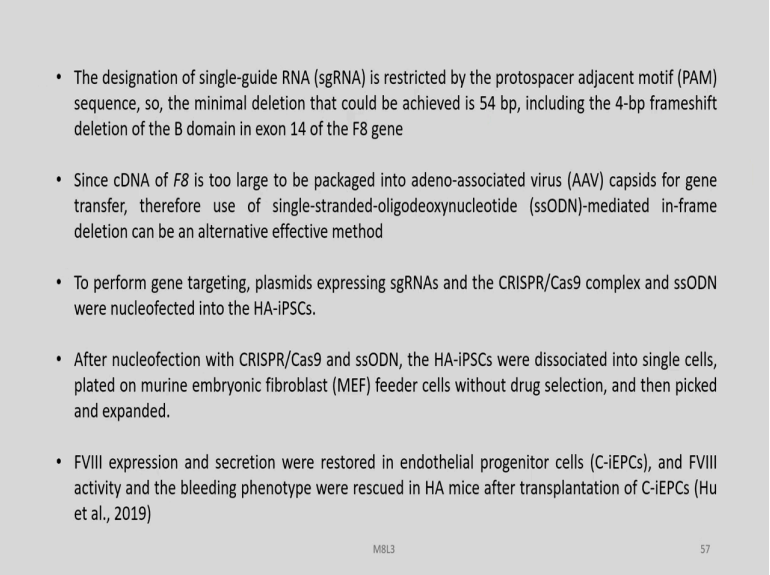

The designation of single-guide RNA is restricted by the protospacer adjacent motif, so the minimal deletion that could be achieved is 54 base pairs, including the 4 base pair frameshift deletion of the B domain in exon 14 of the F8 gene. Since, cDNA of F8 is too large to be packaged into adeno-associated virus capsids for gene transfer, therefore, use of single-stranded-oligodeoxynucleotide mediated in-frame deletion can be an alternative effective method.

To perform gene targeting, plasmids expand expressing sgRNAs and CRISPR Cas9 complex and ssODN were nucleofected into the hemophilia A induced pluripotent stem cells. After nucleofection with CRISPR Cas9 and ssODN, the HA-iPSCs were dissociated into single cells, plated on murine embryonic fibroblast feeder cells without drug selection and then picked and expanded.

Factor VIII expression and secretion were restored in endothelial progenitor cells and factor VIII activity and the bleeding phenotype were rescued in hemophilia A mice after transplantation of C-iEPCs.

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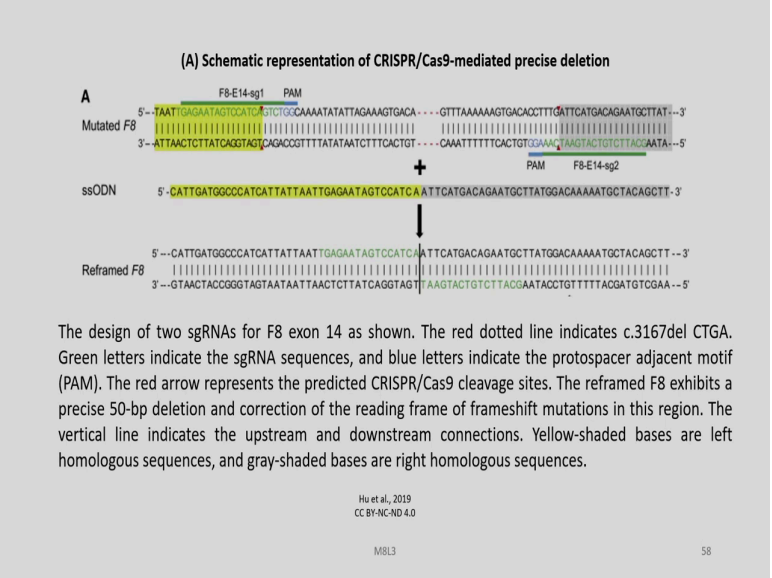

Schematic representation of CRISPR Cas9 mediated precise deletion. The design of two sgRNAs for F8 exon 14 is shown in this figure. The red dotted lines as you can see in the center indicates c.3167del CTGA. The green letters indicate the sgRNA sequences and blue letters indicate the protospacer adjacent motif. The red arrows represent predicted CRISPR Cas9 cleavage sites.

The reframed F8 exhibits a precise 50 base pair deletion and correction of the reading frame of frameshift mutations in this region. The vertical line indicates the upstream and downstream connections. Yellow-shaded bases are left homologous sequences and gray shaded bases are right homologous sequences.

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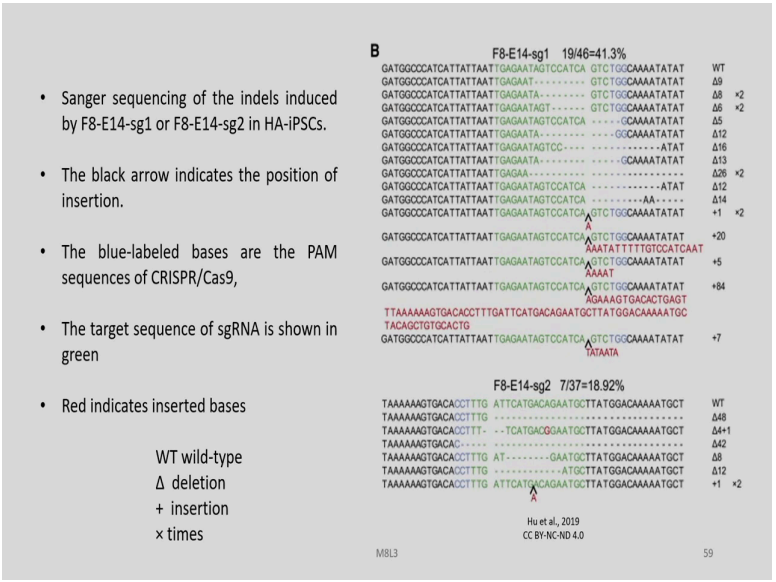

Here is the results of the Sanger sequencing of the indels induced by F8-E14-sg1 or F8-E14-sg2 in hemophilia A iPSCs. The black arrow indicates the position of insertion. The blue-labeled bases are the PAM sequences of the CRISPR Cas9. The target sequence of sgRNA is shown in green color in the center. Red indicates inserted bases, WT stands for wild-type, delta stands for deletion, plus stands for insertion and the x or cross indicates the times.

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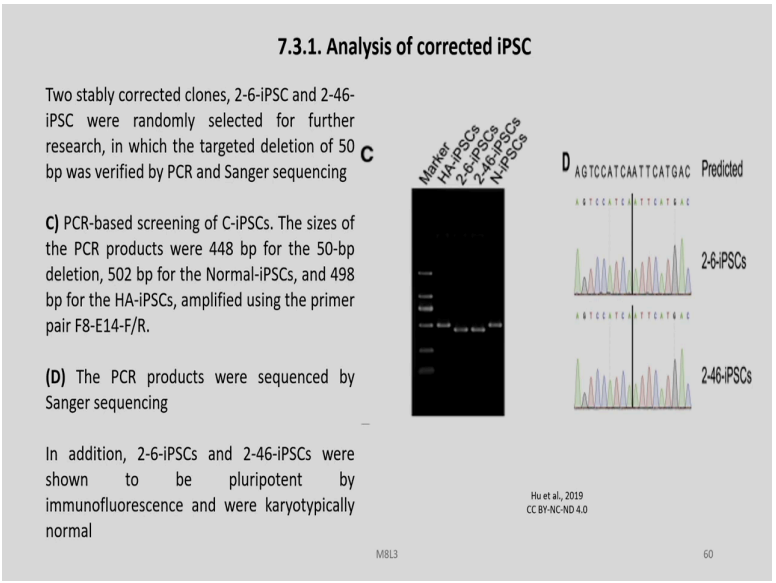

Analysis of corrected iPSCs showed the following results. Two stably corrected clones, 2-6-iPSCs and 2-46-iPSCs were randomly selected for further research, in which the targeted deletion of 50 base pairs was verified by PCR and Sanger sequencing as discussed earlier. In figure C, you can see the PCR based screening of C-iPSCs. The size of the PCR products were around 448 base pairs for the 50 base pair deletion, 502 base pairs for the normal iPSCs and 498 base pairs for the HA-iPSCs, amplified using the primary pair F8-E14-F/R.

In figure D, we can see the PCR products sequenced by Sanger sequencing. In addition 2-6-iPSCs and 2-46-iPSCs were shown to be pluripotent by immunofluorescence and were karyotypically normal.

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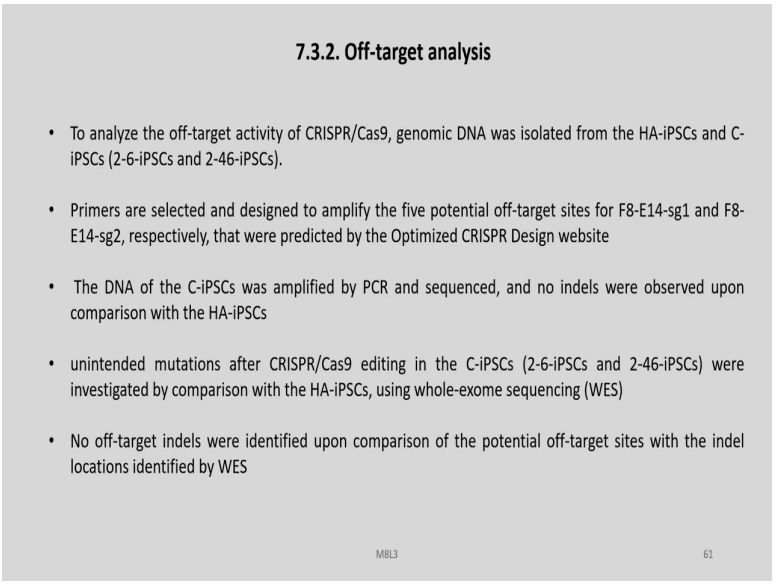

There was also concern of off target hits and off target analysis was carried out. To analyze the off-target activity of CRISPR Cas 9 genomic DNA is isolated from the HA-iPSCs and C-iPSCs about 2-6-iPSCs and 2-46-iPSCs. Primers were selected and designed to amplify the five potential off target sites for F8- E14 and sg 1 and F8-E-14 and sg2 respectively that were predicted by the optimized crisper design website.

The DNA of the C-iPSCs was amplified by PCR and sequenced and no indels were observed upon comparison with the HA-iPSCs. Unintended mutations after CRISPR Cas9 editing in the C-iPSCs were investigated by comparison with the HA- iPSCs, using whole-exome sequencing. In brief no off-target indels were identified upon comparison of the potential off

target sites with the indel locations identified by WES. Therefore, CRISPR Cas9 as a gene editing tool has large application potentials in the treatment of hemophilia.

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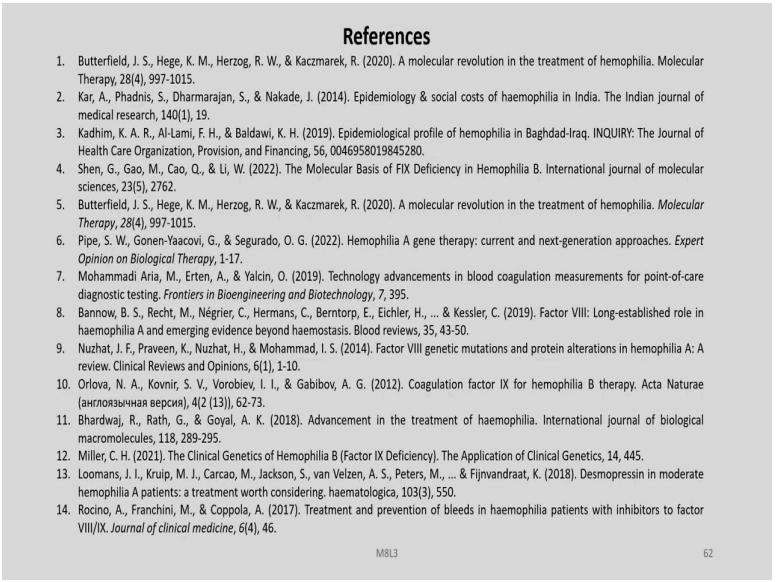

References

1. Butterfield, J. S., Hege, K. M., Herzog, R. W., & Kaczmarek, R. (2020). A molecular revolution in the treatment of hemophilia. Molecular Therapy, 28(4), 997-1015.
2. Kar, A., Phadnis, S., Dharmarajan, S., & Nakade, J. (2014). Epidemiology & social costs of haemophilia in India. The Indian journal of medical research, 140(1), 19.
3. Kadhim, K. A. R., Al-Lami, F. H., & Baldawi, K. H. (2019). Epidemiological profile of hemophilia in Baghdad-Iraq. INQUIRY: The Journal of Health Care Organization, Provision, and Financing, 56, 0046958019845280.
4. Shen, G., Gao, M., Cao, Q., & Li, W. (2022). The Molecular Basis of FIX Deficiency in Hemophilia B. International journal of molecular sciences, 23(5), 2762.
5. Butterfield, J. S., Hege, K. M., Herzog, R. W., & Kaczmarek, R. (2020). A molecular revolution in the treatment of hemophilia. *Molecular Therapy*, *28*(4), 997-1015.
6. Pipe, S. W., Gonen-Yaacovi, G., & Segurado, O. G. (2022). Hemophilia A gene therapy: current and next-generation approaches. *Expert Opinion on Biological Therapy*, 1-17.
7. Mohammadi Aria, M., Erten, A., & Yalcin, O. (2019). Technology advancements in blood coagulation measurements for point-of-care diagnostic testing. *Frontiers in Bioengineering and Biotechnology*, *7*, 395.
8. Bannow, B. S., Recht, M., Négrier, C., Hermans, C., Berntorp, E., Eichler, H., ... & Kessler, C. (2019). Factor VIII: Long-established role in haemophilia A and emerging evidence beyond haemostasis. Blood reviews, 35, 43-50.
9. Nuzhat, J. F., Praveen, K., Nuzhat, H., & Mohammad, I. S. (2014). Factor VIII genetic mutations and protein alterations in hemophilia A: A review. Clinical Reviews and Opinions, 6(1), 1-10.
10. Orlova, N. A., Kovnir, S. V., Vorobiev, I. I., & Gabibov, A. G. (2012). Coagulation factor IX for hemophilia B therapy. Acta Naturae (англоязычная версия), 4(2 (13)), 62-73.
11. Bhardwaj, R., Rath, G., & Goyal, A. K. (2018). Advancement in the treatment of haemophilia. International journal of biological macromolecules, 118, 289-295.
12. Miller, C. H. (2021). The Clinical Genetics of Hemophilia B (Factor IX Deficiency). The Application of Clinical Genetics, 14, 445.
13. Loomans, J. I., Kruip, M. J., Carcao, M., Jackson, S., van Velzen, A. S., Peters, M., ... & Fijnvandraat, K. (2018). Desmopressin in moderate hemophilia A patients: a treatment worth considering. haematologica, 103(3), 550.
14. Rocino, A., Franchini, M., & Coppola, A. (2017). Treatment and prevention of bleeds in haemophilia patients with inhibitors to factor VIII/IX. *Journal of clinical medicine*, *6*(4), 46.

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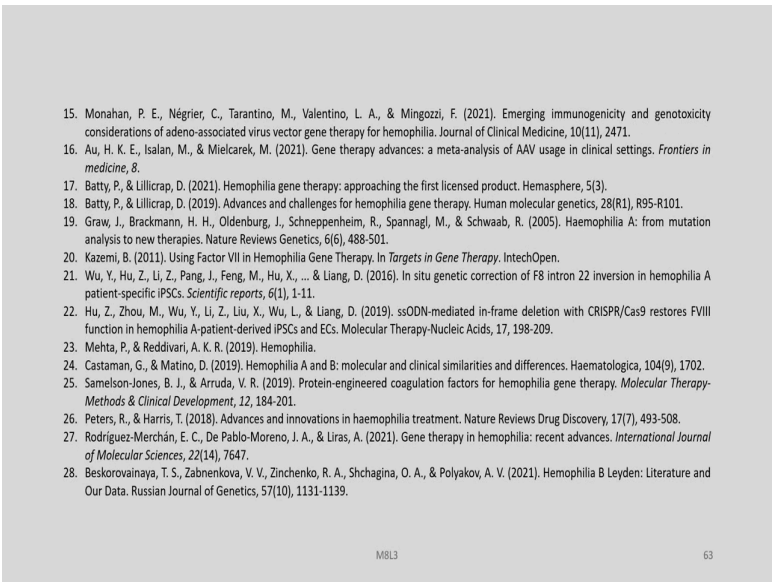

15. Monahan, P. E., Négrier, C., Tarantino, M., Valentino, L. A., & Mingozzi, F. (2021). Emerging immunogenicity and genotoxicity considerations of adeno-associated virus vector gene therapy for hemophilia. Journal of Clinical Medicine, 10(11), 2471.
16. Au, H. K. E., Isalan, M., & Mielcarek, M. (2021). Gene therapy advances: a meta-analysis of AAV usage in clinical settings. *Frontiers in medicine*, *8*.
17. Batty, P., & Lillicrap, D. (2021). Hemophilia gene therapy: approaching the first licensed product. Hemasphere, 5(3).
18. Batty, P., & Lillicrap, D. (2019). Advances and challenges for hemophilia gene therapy. Human molecular genetics, 28(R1), R95-R101.
19. Graw, J., Brackmann, H. H., Oldenburg, J., Schneppenheim, R., Spannagl, M., & Schwaab, R. (2005). Haemophilia A: from mutation analysis to new therapies. Nature Reviews Genetics, 6(6), 488-501.
20. Kazemi, B. (2011). Using Factor VII in Hemophilia Gene Therapy. In *Targets in Gene Therapy*. IntechOpen.
21. Wu, Y., Hu, Z., Li, Z., Pang, J., Feng, M., Hu, X., ... & Liang, D. (2016). In situ genetic correction of F8 intron 22 inversion in hemophilia A patient-specific iPSCs. *Scientific reports*, *6*(1), 1-11.
22. Hu, Z., Zhou, M., Wu, Y., Li, Z., Liu, X., Wu, L., & Liang, D. (2019). ssODN-mediated in-frame deletion with CRISPR/Cas9 restores FVIII function in hemophilia A-patient-derived iPSCs and ECs. Molecular Therapy-Nucleic Acids, 17, 198-209.
23. Mehta, P., & Reddivari, A. K. R. (2019). Hemophilia.
24. Castaman, G., & Matino, D. (2019). Hemophilia A and B: molecular and clinical similarities and differences. Haematologica, 104(9), 1702.
25. Samelson-Jones, B. J., & Arruda, V. R. (2019). Protein-engineered coagulation factors for hemophilia gene therapy. *Molecular Therapy-Methods & Clinical Development*, *12*, 184-201.
26. Peters, R., & Harris, T. (2018). Advances and innovations in haemophilia treatment. Nature Reviews Drug Discovery, 17(7), 493-508.
27. Rodríguez-Merchán, E. C., De Pablo-Moreno, J. A., & Liras, A. (2021). Gene therapy in hemophilia: recent advances. *International Journal of Molecular Sciences*, *22*(14), 7647.
28. Beskorovainaya, T. S., Zabnenkova, V. V., Zinchenko, R. A., Shchagina, O. A., & Polyakov, A. V. (2021). Hemophilia B Leyden: Literature and Our Data. Russian Journal of Genetics, 57(10), 1131-1139.

With this we come to end of this lecture, these are some of the references from which we have taken materials for preparing this particular lecture.

And thank you for your patient hearing.