The liver takes a leading role in the hemophilia gene therapy story

Gene therapies for hemophilia are currently being studied to determine their safety and efficacy. Approved gene therapies for hemophilia may have different labeling in different countries.

Pfizer

Contents

> Synopsis

The liver is the primary site of FVIII and FIX protein production. This, in addition to the unique anatomical properties of the liver, makes it the ideal target organ for hemophilia gene therapies.[1–3] There are several types of vectors , but recombinant adeno-associated virus (rAAV) vectors are used in most clinical trials to achieve targeted delivery of the transgene to the liver.[4] Continued research around AAV serotypes and tropism has led to the adoption of approaches to improve liver tropism.[5,6] Additionally, the tolerogenic nature of the liver can be exploited to support transgene expression, potentially increasing the levels of coagulation factors without eliciting a detrimental immune response.[7] Several unknowns associated with liver-directed hemophilia gene therapies remain and require further research and long-term data collection.[8]

The information included in this brochure is accurate as of August 2023. Check back regularly for further updates.

Basic physiology of the liver: hepatology 101

Anatomy of the liver

> The liver is a large complex organ composed of several different cell types, each with unique functions. Together, these cells regulate hepatic function at multiple levels[9,10]

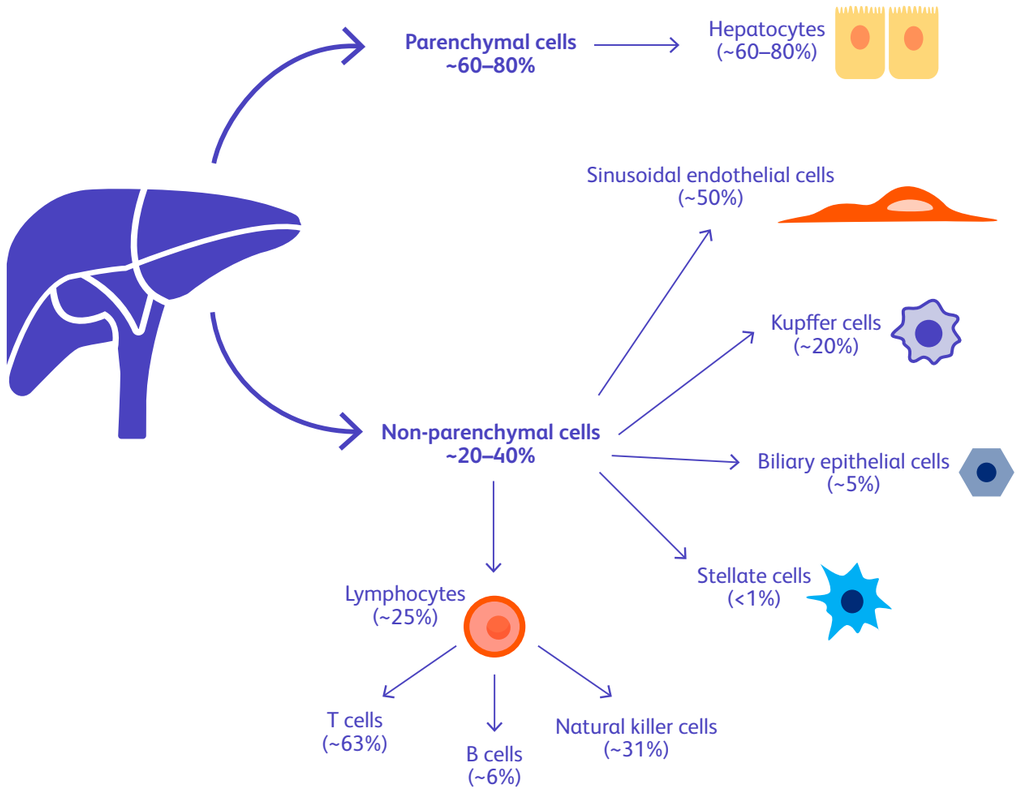


Figure developed from Racanelli V, Rehermann B. 2006,[11] Banales JM. 2019,[12] Maepa SW, Ndlovu H. 2020[13] and Gonzalez-Sanchez E, et al. 2017.[14]

The liver is highly vascularized with a dual blood supply, receiving 25% of the cardiac output[10,15,16]

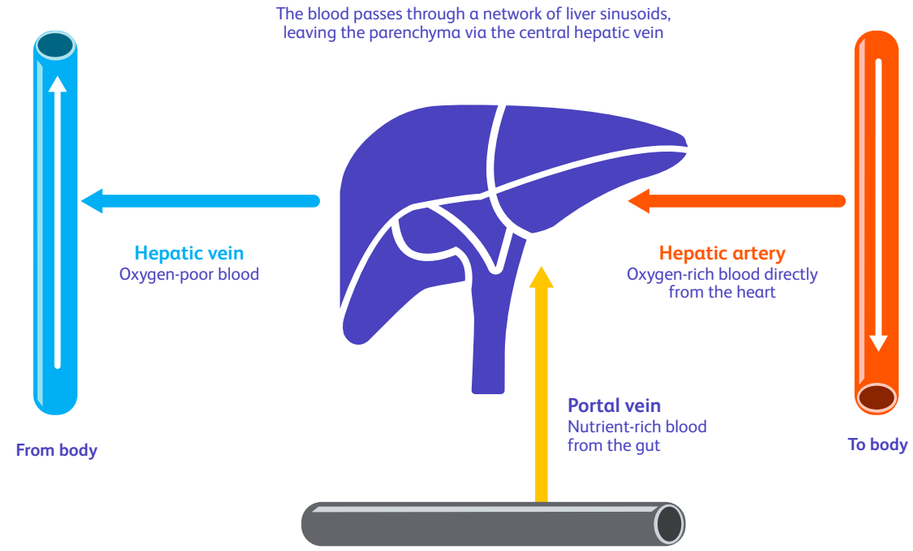


Figure adapted from Racanelli V, Rehermann B. 2006.[11]

Liver growth and development occur throughout childhood[17]

> The human liver doubles in weight at 4 months, 16 months, 6 years and 12 years[17,18]

> As one approaches adulthood, hepatocyte cell turnover transitions from a high to a low rate[19]

> The adult liver is 16 times heavier than the neonatal liver[17]

> Under normal conditions, the adult liver has very little proliferative activity[20]

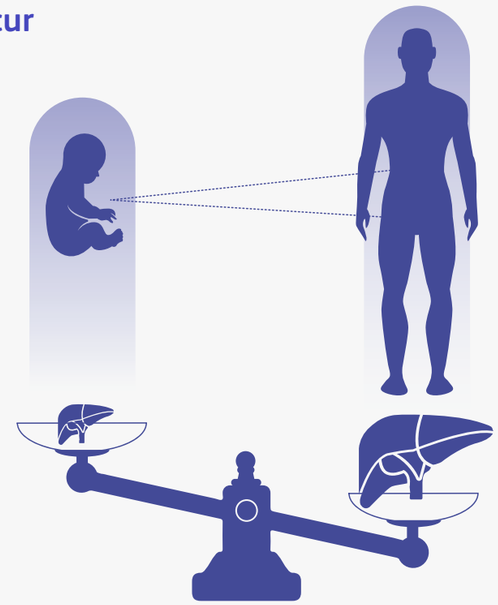


Figure developed from Trefts E, et al. 2017,[9] Nguyen TH, Ferry N. 2004,[10] and Kalra A, et al. 2020.[15]

Liver involvement in cellular processes

The liver is involved in a range of natural cellular processes, including:

- Blood volume regulation and blood detoxification[9,10,15]
- Immune system support[9,15]
- Processing, partitioning and metabolism of macronutrients[9,10]
- Protein synthesis (including synthesis of coagulation factors)[15]

Liver enzymes

The involvement of the liver in cellular processes is facilitated by a myriad of enzymes.[21] Both the detection and level of these liver enzymes can reflect liver health.[21,22] Biochemical abnormalities in many of these liver enzymes can be assessed using liver function tests, and may indicate complications or damage to the hepatic system.[21,22]

Liver health can be measured by various methods, including enzyme tests, biochemical markers and other non-invasive techniques, and biopsies[23,24]

Liver health assessment parameter	Examples	Indication of liver health
Liver enzyme tests[23]	> Alanine aminotransferase (ALT)[23] > Aspartate aminotransferase (AST)[23] > Alkaline phosphatase[23]	Elevation above normal ranges may be indicative of liver injury[23]
Biochemical markers[23,24]	> Albumin[23] > Bilirubin[23] > Biomarkers associated with fibrosis[24]	Markers of hepatocellular function[23] or fibrosis[24]
Other non-invasive techniques[24]	> Ultrasonographic or magnetic resonance elastography[24]	Measurement of liver stiffness used to diagnose and stage fibrosis[24]
Liver biopsy[23–26]	> Percutaneous, laparoscopic or transjugular liver biopsy[25,26]	Gold standard to assess liver fibrosis[24] and confirm diagnoses[23]

The liver as a tolerogenic organ

- The liver is a lymphoid organ and is often termed an ‘immunogenic’ or ‘tolerogenic’ organ due to its many unique immunologic properties[27,28]
- The tolerance effect of the liver was first evidenced in a pig model of transplantation, where despite a major histocompatibility complex (MHC) mismatch, the liver readily accepted the allograft[27,29]

What makes the liver a tolerogenic organ?

The liver has many unique immunologic properties[27,28]

- Induction of immune tolerance[27,28]
- Strong innate immunity[27,28]
- Poor adaptive immune response:[27,28] under basal conditions, liver-resident cells functionally suppress the adaptive immune response, maintaining a state of immune unresponsiveness[30]

The cell types that make up the liver most likely act synergistically to skew the immune responses toward tolerance[29]

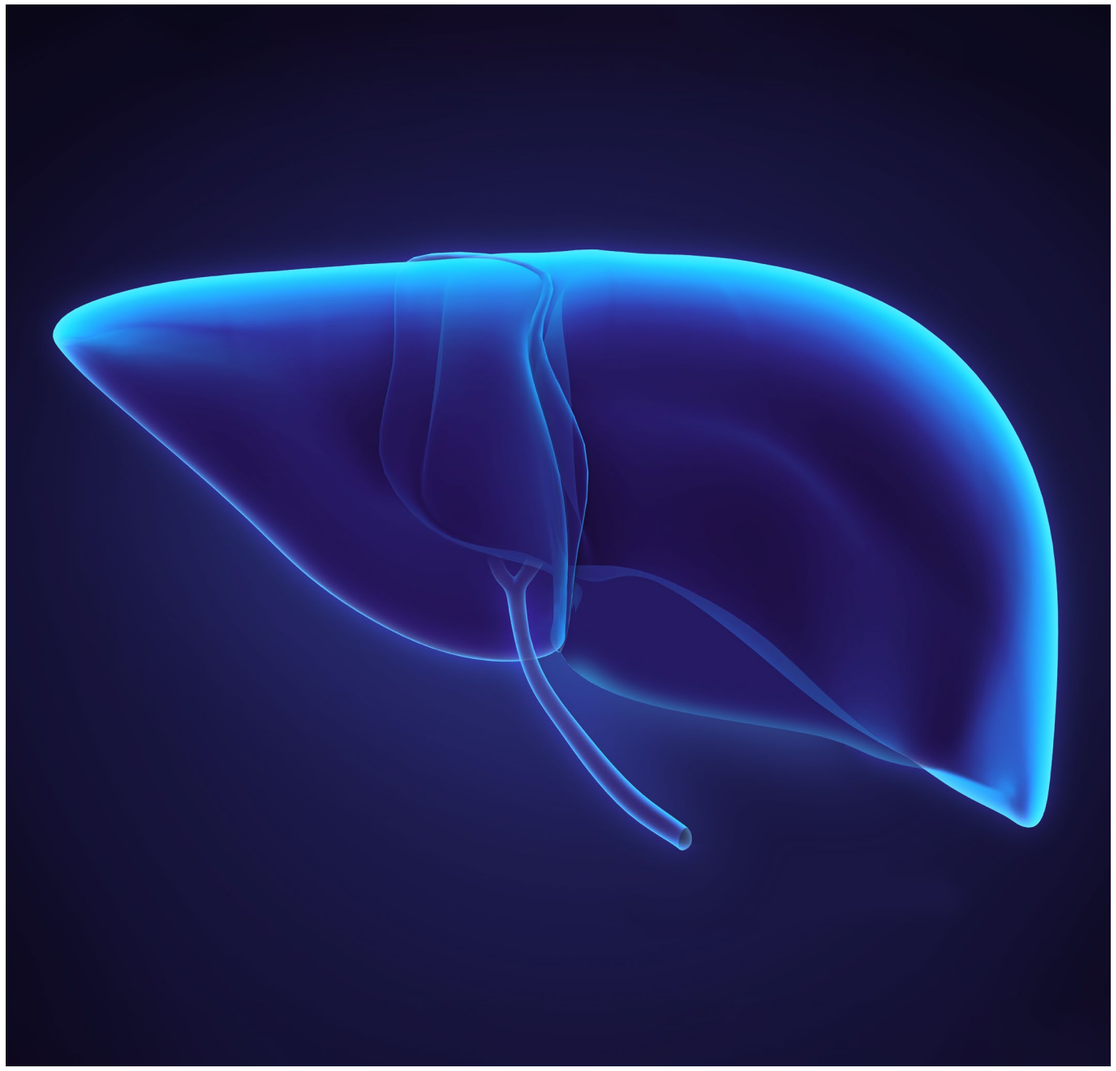
Able to efficiently and rapidly protect itself from potentially toxic agents without generating an immune response[11,28,31]

- Exposed to high levels of foreign antigens from the digestive tract[11,28,31]
- Immunotolerance enables the liver to avoid generating a detrimental innate response to exposure to these antigens[11,28,31]

Mechanisms underlying the tolerogenic nature of the liver

The following are the mechanisms underlying the tolerogenic nature of the liver:

- Intrahepatic innate immune cells express low or undetectable levels of the MHC antigens, costimulatory molecules and other effector molecules, making it difficult for them to induce an innate or adaptive immune response[32]
- Innate immune cells of the liver (such as Kupffer cells)[33] immunosuppress functions of other intrahepatic cells by direct contact or through the secretion of cytokines, such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β)[32]



The role of the liver in coagulation / hemostasis

- The liver is a key organ for most metabolic pathways; numerous inherited diseases have their origin in this organ, including **hemophilia A and B**[10]
- The liver is the primary site of synthesis of nearly all coagulation factors (with the exception of von Willebrand factor[25]) and also several proteins that are involved in fibrinolysis and anticoagulation[2]

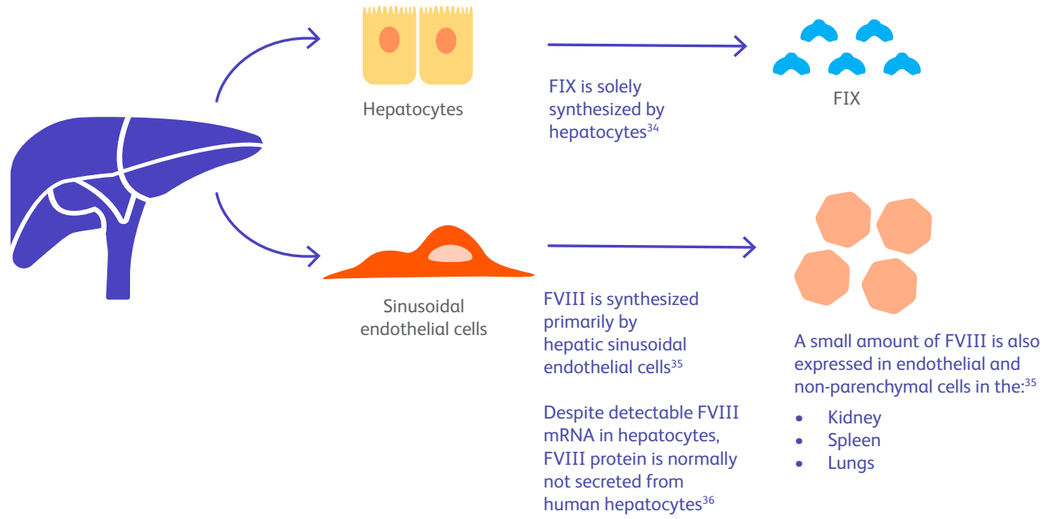


Figure developed from Verdera HC, et al. 2020.[37]

Potential targets for liver-directed gene therapy

A number of monogenic liver-related conditions may benefit from liver-directed gene therapy, including the following: [3,17]

- Alpha-1 antitrypsin deficiency
- Crigler-Najjar
- Hemophilia A
- Hemophilia B
- Homozygous familial hypercholesterolemia
- Ornithine transcarbamylase deficiency
- Wilson disease

The liver is a target for gene therapy in patients with hemophilia, as this is where clotting factors are normally expressed. However, for patients with underlying liver disease and comorbid conditions, such as viral hepatitis, it is important to also consider alternative, ectopic target tissues or alternative treatment approaches.[1]

Liver hepatocytes are the ideal target for hemophilia gene therapy

FVIII and FIX are naturally produced in the liver[1]

Post-translational modifications needed to process FIX produced via gene therapy innately follow the normal pathways in hepatocytes[1]

Rapid accumulation of viral particles: unique dual blood supply of the liver means that, at any given time, it receives 10–15 % of the total blood volume, leading to rapid accumulation of viral particles within the liver post administration[3]

Efficient transduction: specialized fenestrated endothelium along the hepatic sinusoids allows for vector particles to pass from the blood to the hepatocytes, making transduction of cells more efficient than in organs with continuous endothelium[3]

Minimal dilution effects: under normal conditions, most cells in a fully mature liver are quiescent (only 1–2 % of hepatocytes turning over at any given time),[3] with an average lifespan of 200–300 days, so gene therapy administered to these cells undergoes minimal dilutional effects[3]

Hemophilia A and B are well-characterized diseases, each caused by a mutation in a single gene (*F8* and *F9*, respectively) that results in the lack of a single protein, making it a suitable target for gene therapy.[38]

Liver-directed rAAV vector-mediated hemophilia gene therapy

rAAV vectors are currently the vector of choice for hemophilia gene therapies under investigation[4,8]

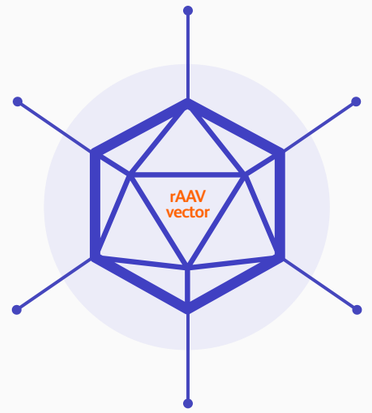
Early rAAV vector-mediated gene therapy studies for hemophilia B targeted easily accessible skeletal muscle. Although skeletal muscle does not normally express FVIII or FIX, it does have robust secretory capacity and the ability to post-translationally modify the protein, allowing for the production of functional protein.[1]

- However, these post-translational modifications were shown to be not as efficient as in hepatocytes[1]
- Early animal studies (e.g. mouse models with canine-derived FIX) showed some anti-FIX inhibitor formation and low transduction efficiency with muscle-directed rAAV vector gene therapy[39]
- Subsequent studies pursued investigating liver-directed rAAV vector-mediated gene therapy further[1]

Currently rAAV is the most frequently used vector in hemophilia gene therapy studies[4]

The specific AAV serotypes that support liver transduction include AAV2, AAV5, AAV6, AAV8, AAVrh74 and AAV9[8,40,41]

Different AAV serotypes possess divergent capsid protein amino acid sequences, and these variations influence the tropism of each serotype. Tropism is the level of affinity for particular cell types and organs[42]



AAV can mediate gene transduction in numerous dividing and quiescent cell types[43]

AAV mediates gene transduction without inducing any known pathogenicity[43]

The AAV capsid can be engineered to enhance transduction and minimize immune responses[44]

Optimizing liver-directed rAAV vectors

Capsid sequence variations of different AAV serotypes influence cell tropism.[42,44] The different serotypes are not only liver-specific in their tropism; for example, AAV8 targets cells in the heart and pancreas as well as those in the liver.[40] Therefore, different strategies have been employed to increase liver-specific tropism and to achieve greater control of the transgene expression in liver hepatocytes.

Approaches to improve transduction efficiency and transgene expression include:

- Pseudotyping to enhance liver tropism
- Using liver-specific promoters to amplify transgene expression

Pseudotyping to enhance liver tropism

- Pseudotyping refers to the packaging of the genome from one AAV serotype into the capsid of another AAV serotype to create **hybrid vectors.**[5] Pseudotyping an rAAV vector with components from different AAV serotypes can substantially alter tissue tropism.[5] It is generally carried out with the inverted terminal repeats from the AAV2 serotype packaged in the capsid from a different serotype, which can enhance transduction efficiency and tropism[5,6]
- **Example:** AAV8 has a higher affinity for hepatocytes when compared with AAV2. rAAV2 vectors mediate a persistent high-level expression *in vivo* but have a relatively broad tropism[45]
- rAAV2/8 contains the inverted terminal repeats from AAV2 and the capsid of AAV8
  - rAAV2/8 can transduce 3–4-fold more hepatocytes and deliver 3–4-fold more genomes per transduced cell than AAV2 alone[45]
  - Animal studies have shown the ability of rAAV8-pseudotyped vectors to transduce more than 95% of hepatocytes following a single injection, depending upon the dose[45]

Liver-specific promotors to amplify transgene expression

- The use of small tissue-specific promoters increases tissue-specific transgene expression and in turn increases the packaging capacity of the rAAV expression cassette. In addition, this approach can minimize the cytotoxic T lymphocyte immune response to the rAAV vector.[44] Recently, different liver-specific promoter and enhancer elements were combined to generate small promoters and enhancers for the efficient packaging and transduction of rAAV[44]
- **Example:** Early liver-specific promoters were developed around secreted proteins found within the liver such as human serum albumin and alpha-1-antitrypsin.[3] Nathwani and team used a chimeric promotor of the apolipoprotein E/C-I hepatic control region combined with the human alpha-1-antitrypsin core promoter in their early hemophilia B trials[3,46]

Other considerations for liver-directed gene therapy

Key biochemical markers of liver health observed following liver-directed rAAV vector hemophilia gene therapy

Biochemical marker	Relevance in liver gene therapy	Approaches to overcome elevated levels
Alanine aminotransferase (ALT) > Role in hepatocyte integrity[21]	> Elevation of serum ALT levels (transaminitis): – Elevations above a certain threshold may indicate hepatocellular injury[22] or rAAV-vector-mediated liver toxicity[49] – Has been observed as a vector-related adverse event in rAAV vector gene therapy studies to date[49] – Has been correlated with a loss of FVIII or FIX expression in hemophilia A and B studies[4,49,50]	> Data suggest that transaminitis may be managed with immunosuppressants (e.g. corticosteroids)[4,49,50] – Initiation of steroid treatment appears to prevent loss of protein expression if initiated rapidly after ALT elevation[4,49,50] – Immunosuppressants may not work in all circumstances[6] > The use of prophylactic immunosuppressant regimens following rAAV vector gene therapy infusion is being investigated in certain clinical trials[49,51] > Further study is required to understand the immunomodulatory effects of the various immunosuppressant regimens
Aspartate aminotransferase (AST) > Role in hepatocyte integrity[21]	> Elevation of serum AST levels (transaminitis): – Elevations above a certain threshold may indicate hepatocellular injury[22] – Has been reported less frequently than ALT elevations; however, remains a possible adverse event in hemophilia gene therapy trials to date[49] – Has been correlated with a loss of protein expression in clinical studies[50]	

Of note, some studies administering rAAV2 vector liver-directed hemophilia gene therapy reported some participants developing a cellular immune response against the rAAV capsid, resulting in a decrease in transgene expression.[4,52]

> **This was also associated with an increase in liver enzymes (e.g. ALT and AST)[4,52]**

Long-term monitoring of the liver and carrying out liver function tests after administration of liver-directed rAAV vector-mediated gene therapy is important.

Potential immune responses to AAV vectors

> Natural exposure to AAV during a person's life can result in the development of:
  – AAV-specific neutralizing antibodies (nAbs)[50,53]
  – Non-neutralizing antibodies[50]
> nAbs can bind to capsids and prevent transduction and the expression of the chosen therapeutic gene[17,54]
> The innate immune system may also recognize rAAV vector components or the transgene as foreign material and potentially illicit an immune response[17]
> Of note, pre-existing immunity originating from the exposure to wild-type AAV, can generate both humoral and cell-mediated immunity to the virus, which can cross-react with rAAV vectors; this, therefore, may present a barrier to successful gene transfer[37]

Implications of continued growth of the liver in children as it applies to hemophilia gene therapy

> Liver cells divide rapidly during childhood; the human liver doubles in weight at 4 months, 16 months, 6 years and 12 years.[17] Therefore, non-integrating transgenes will eventually be diluted, leading to the progressive loss of transgene expression and subsequent reduction in the levels of the FVIII and FIX proteins[17,55,56]

Liver toxicity

Liver disease and potential implications for hemophilia gene therapy

> Risk factors and diseases can cause chronic liver disease in the general population; for example:[26]
  – Toxins (e.g. chemicals, drugs)
  – Alcohol abuse for a prolonged time
  – Infection (e.g. hepatitis B and C)
  – Autoimmune diseases
  – Genetic and metabolic disorders (e.g. non-alcoholic steatohepatitis [NASH])
  – Prolonged or high doses of hepatotoxic medications (e.g. acetaminophen)
> Currently, hemophilia gene therapy clinical trials exclude patients with underlying liver conditions[7]
> Safety of liver-directed gene therapy in those with underlying liver conditions is unknown[57]

Selected known unknowns of liver-directed hemophilia gene therapy

Impact of alcohol consumption

- Excessive alcohol can lead to the destruction of liver cells and tissue scarring through alcoholic hepatitis or cirrhosis[58]
- The impact of alcohol consumption on long-term transgene expression in the liver post gene therapy is currently unknown[59]
- The Medical and Scientific Advisory Council (MASAC) recommends that the consumption of hepatoxic agents such as alcohol should be carefully evaluated, especially during early timepoints, following administration of rAAV vectors[60]

Considerations for hemophilia A and the cell type targeted

- FVIII is not normally secreted from human hepatocytes, and therefore gene delivery to these cells may be limited by the intrinsic inability of the human hepatocyte to fully synthesize and secrete FVIII, despite the presence of mRNA[36,61]
  - However, this does not prevent gene delivery to the hepatocytes[61]
- The long-term expression of FVIII in hepatocytes is unknown and will require long-term monitoring[61]

Possible consequences of genome integration

- rAAV vector-delivered transgenes are **predominantly** non-integrating, meaning that the delivered transgene exists as an episome outside the patient's DNA[6,56]
  - Random integration events have been observed with a low frequency (0.1–1 % of transduction events)[6]
- Uncertainties remain around the long-term effects of rAAV vector liver-directed gene therapy[62]
  - MASAC published a recommendation to study sponsors and investigators to incorporate liver biopsies to examine longitudinal assessment of liver histology, viral capsid protein presence, inflammation and immune markers, and distribution of transgene and therapeutic protein across cell types and regions in the liver[62]
- Early analyses from the first-in-human liver biopsy study following gene therapy for hemophilia A were recently shared[63]
  - Histopathology and vector DNA form were evaluated[63]
  - Demonstrated persistent FVIII expression consistent with the presence of circularized, full-length rAAV vector DNA in human liver[63]
- Hepatocellular carcinoma (HCC) was observed in neonatal mice treated using rAAV vector associated with transgene integration into the *Rian* locus (a region not present in the human genome)[4,64]
  - No cancer development has been observed in older rodents or non-human primate models to date[4]
  - A 10-year follow-up study in dogs demonstrated rAAV integration and clonal expansion had occurred in the liver cells post-administration, but there was no evidence of tumorigenesis[65]
- In humans, the long-term risk of genotoxicity following rAAV-delivered gene therapy remains unknown[65]
- Long-term follow-up will be important to monitor for, and improve our understanding of, potential genotoxic occurrences

References

1. Chuah MK, et al. *J Thromb Haemost* 2013:11(1):99–110.
2. Kopec AK, et al. *Thromb Res* 2014:133(Suppl. 1);S57–9.
3. Kattenhorn LM, et al. *Hum Gene Ther* 2016;27(12):947–61.
4. Doshi BS, et al. *Ther Adv Hematol* 2018:9;273–93.
5. Balakrishnan B, Jayandharan GR. *Curr Gene Ther* 2014;14:1–15.
6. Colella P, et al. *Mol Ther Methods Clin Dev* 2018;8:87–104.
7. Samelson-Jones BJ, Arruda VR. *Front Immunol* 2020;11:618.
8. George LA. *Blood Adv* 2017;1(26):2591–9.
9. Trefts E, et al. *Current Biol* 2017:27;1141–55.
10. Nguyen TH, Ferry N. *Nat Gene Therapy* 2004;11:S76–S84.
11. Racanelli V, Rehermann B. *Hepatology* 2006;43:S54–62.
12. Banales JM, et al. *Nat Rev Gastroenterol Hepatol* 2019;16(5):269–81.
13. Maepa SW, Ndlovu H. *Stem Cells* 2020;38(5);606–12.
14. Gonzalez-Sanchez E, et al. *Biochim Biophys Acta Mol Basis Dis* 2017;1863(7):1699–708.
15. Kalra A, et al. *Physiology, Liver*. In: StatPearls [Internet]. Treasure Island (FL), StatPearls Publishing, 2020.
16. Lautt WW. *Hepatic Circulation: Physiology and Pathophysiology*. San Rafael (CA), Morgan & Claypool Life Sciences, 2009.
17. Baruteau J, et al. *J Inherit Metab Dis* 2017;40(4):497–517.
18. Coppoletta JM, Wolbach SB. *Am J Pathol* 1933;9(1):55–70.
19. Pipe SW. *Haemophilia* 2021;27(Suppl. 3):114–21.
20. Ramboer E, et al. *J Hepatol* 2014:61(4):925–43.
21. Giannini EG, et al. *CMAJ* 2005;172(3):367–79.
22. Kasarala G, Tillmann HL. *Clin Liver Dis* 2016;8(1):13–8.
23. Kwo P et al. *Am J Gastroenterol* 2017;112(1):18–35.
24. Afdhal NH. *Gastroenterol Hepatol* 2012;8(9):605–7.
25. Burroughs A, Senzolo M. www.cancertherapyadvisor.com/home/decision-support-in-medicine/hematology/coagulopathy-inliverdisease/ (Accessed August 2023).
26. Sharma A, Nagalli S. Chronic Liver Disease. [Updated 2020 Feb 28]. In: StatPearls [Internet]. Treasure Island (FL), StatPearls Publishing, 2020.
27. Horst AK, et al. Cell Mol Immunol 2016;13:277–92.
28. Gao B. *Cell Mol Immunol* 2016;13:265–6.
29. Sack BK, et al. *Mol Ther* 2014;1:14013.
30. Kubes P, Jenne C. *Annu Rev Immunol* 2018;36:247–77.
31. Robinson MW et al. *Cell Molec Immunol* 2016;13:267–76.
32. Huang H, et al. *Front Immunol* 2018;9:2401.
33. Dixon LJ, et al. *Compr Physiol* 2013;3:785–97.
34. Tatsumi K, et al. *Cell Med* 2012;3:25–31.
35. Zanolini D, et al. *Haematologica* 2015;100(7):881–92.
36. Arruda VR. *Haematologica* 2015;100:849–50.
37. Verdera HC, et al. *Mol Ther* 2020;28:723–46.
38. Lheriteau E, et al. *Blood Rev* 2015;29(5):321–8.
39. Wang L, et al. *J Thromb Haemost* 2011;(9)10:2009–19.
40. Wu Z, et al. *Mol Ther* 2006;14:316–27.
41. Mingozzi F, et al. *J Virol* 2002;76:10497–502.
42. Holehonnur R, et al. *BMC Neuro* 2014;15:28–42.
43. Ellis BL, et al. *Virology J* 2013;10:74–84.
44. Li C, Samulski JR, et al. *Nat Gen Rev* 2020;21:255–72.
45. Sands MS. *Methods Mol Biol* 2011;807:147–57.
46. Nathwani AC, et al. *Blood* 2006;107(7):2653–61.
47. Crispe IN. *Nat Rev* 2003;3:51–62.
49. Pipe S, et al. *Mol Ther Methods Clin Dev* 2019;15:170–8.
50. Mingozzi F, High KA. *Blood* 2013;122:23–36.
51. Chowdary P, et al. *Blood* 2018;132(Suppl. 1):631.
52. Manno CA, et al. *Nat Med* 2006;12(3)342–7.
53. Scallan CD, et al. *Blood* 2006;107:1810–7.
54. Rapti K, et al. *Mol Ther* 2012;20:73–83.
55. NHF feature: www.hemophilia.org/sites/default/files/NHF_Winter%2020_Feature_GeneTherapyNovelTechWorkshop.pdf (Accessed August 2023).
56. Naso MF, et al. *BioDrugs* 2017;31:317–34.
57. George LA. *Hematology Am Soc Hematol Educ Program* 2017;587–94.
58. Osna NA, et al. *Alcohol Res*. 2017;38(2):147–61.
59. Sidonio RF, et al. *Blood Rev* 2021;47:100759.
60. MASAC Document 254, 2018. www.hemophilia.org/sites/default/files/document/files/254-gene-therapy-trials.pdf (Accessed August 2023).
61. Lange AM, et al. *Mol Ther Methods Clin Dev* 2016;3:16064.
62. MASAC Document 256, 2019. www.hemophilia.org/sites/default/files/document/files/256.pdf (Accessed August 2023)
63. Fong S, et al. *Res Pract Thromb Haemost* 2020;4(Suppl 1):OC03.4.
64. Donsante A, et al. *Science* 2007;317:477.
65. Nguyen GN et al. *Nat Bio* 2020;39:47–55.