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Review Article

Liver health in hemophilia in the era of gene therapy

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ABSTRACT

Gene therapy for hemophilia is a groundbreaking treatment approach with promising results and potential to reduce the burden of the disease. However, uncertainties remain, particularly regarding the liver side effects of AAV gene therapy, which are more common in hemophilia A.

Unlike some other diseases, such as spinal muscular atrophy, where the target cell for gene therapy is different from the one affected by side effects, hemophilia gene therapy operates within the same cellular domain-the hepatocyte. This overlap is challenging and requires a targeted strategy to mitigate the risks associated with liver injury, which often requires temporary immunosuppressive therapy. A comprehensive approach is essential to increase the efficacy of gene therapy and reduce the likelihood of hepatocyte damage. Key components of this strategy include a thorough pre-gene therapy assessment of liver health, careful post-gene therapy liver monitoring, and prompt therapeutic intervention for loss of transgene expression and liver injury. Collaboration between hematologists and hepatologists is essential to ensure a well-coordinated management plan for patients undergoing hemophilia gene therapy.

This review addresses the critical aspect of hepatic comorbidities in patients with hemophilia, emphasizing the need to identify and address these issues prior to initiating gene therapy. It examines the known mechanisms of liver damage and emphasizes the importance of liver monitoring after gene therapy. In addition, the review draws insights from experiences with other AAV-based gene therapies, providing valuable lessons that can guide hemophilia centers in effectively managing liver damage associated with hemophilia gene therapy.

1. Introduction

Hemophilia is a rare X-linked inherited disorder caused by a deficiency of either factor VIII (hemophilia A) or factor IX (hemophilia B). The liver, specifically hepatocytes, is crucial for synthesizing these coagulation factors, while hepatic sinusoidal endothelial cells produce factor VIII. Many patients with hemophilia (PWH) born before 1985 faced liver health challenges and were exposed to hepatitis B and C viruses through plasma-derived replacement therapies and blood transfusions [1]. Today, these patients over the age of 40 may be candidates for hemophilia gene therapy (GT). However, those born after 1985 have been spared such exposure due to advances in treatment safety. PWH generally have lower physical activity and more sedentary lifestyles than non-hemophilic peers [2], a trend exacerbated in developing countries without early prophylaxis [3]. Sedentary lifestyles can lead to obesity and hepatic steatosis [2,4]. Chronic pain from hemophilic arthropathy often requires analgesics [5], posing hepatotoxic risks. GT using AAV technology targets hepatocytes and may induce liver toxicity, requiring careful assessment of liver function and monitoring of PWH before and after GT. Healthcare professionals at hemophilia treatment centers, as well as hepatologists, must have a thorough understanding of the essential role of liver health in patients considered for GT. Liver disease is the second leading cause of years of working life lost in Europe, after ischemic heart disease [6]. Excessive alcohol consumption, ultra-processed foods and obesity are key factors in liver-related health problems. Early detection and management of liver disease in hemophiliacs is critical to minimizing its impact.

As innovative hemophilia therapies that target hepatocytes become

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more widely available, collaboration between hematologists and hepatologists is expected to increase. This collaboration is particularly important not only in the management of hepatitis B and C infections in PWH, but also in the selection of patients for GT, the follow-up after GT with regard to the risk of liver toxicity during the first year and the theoretical long-term risk of developing a hepatocellular carcinoma (HCC).

This article reviews common liver diseases in PWH, highlighting the need for their thorough screening and management before GT. It also discusses multidisciplinary post-GT monitoring to minimize liver toxicity and maximize therapeutic efficacy. The overarching goal is to optimize hemophilia care, improve GT outcomes, and protect the liver from potential toxicities associated with this innovative treatment.

2. Viral hepatitis and hemophilia

Despite a decline in HIV-related deaths since the 1990s [7], viral infections remained a significant mortality risk for PWH until the early 2000s [8]. Hepatitis C complications, including severe cirrhosis, liver failure and HCC, were the leading causes of death in this population. Proactive screening for HCV infection, such as testing for serum anti-HCV antibodies, remains critical for this population in countries where blood products are still widely used [9]. Acute HCV infection is often asymptomatic, leading to delayed diagnosis, with spontaneous resolution occurring in 10–20 % of cases [10]. HCV genotype 1 carries a higher risk (65–70 %) of chronic hepatitis than genotypes 2 or 3. Chronic hepatitis C (CHC) progresses slowly, with 10 % of patients potentially developing complications of liver cirrhosis after 10 to 20 years [11]. Co-infection with HCV and HIV accelerates liver disease progression. Due to the risk of liver fibrosis, cirrhosis (observed in 30–35 % of cases), liver failure [12] and HCC development, PWH and chronic HCV infection should be managed by hepatologists [13,14].

Direct-acting antivirals (DAAs) have revolutionized the treatment of chronic HCV. With 98–99 % success rates and 8 to 12-week durations in real-life studies the new generation of DAA combinations sofosbuvir + velpatasvir and glecaprevir + pibrentasvir are moving Europe towards hepatitis C elimination [15]. A study of 200 chronic HCV-infected PWH demonstrated viral clearance in at least 95 % of cases, regardless of HCV genotype or liver lesion severity, and allowed for the rescue of patients who had previously failed first generation DAAs [16]. Of note, health-related quality of life is lower in in HCV-cured PWH compared to those who have never been chronically infected with HCV, supporting the notion that a psychosocial follow-up and support are indicated [17].

While reducing long-term complications such as liver failure, the impact on HCC in PWH remains debated. Some studies show reduced incidence, while others with longer follow-up show no reduction in HCC incidence [18–20]. Large-scale studies in the general population of CHC patients show a reduced risk of cirrhosis, HCC, and overall mortality [21]. However, the risk of HCC remains, especially in patients with advanced liver fibrosis and co-morbidities. Therefore, regular monitoring is mandatory for all hemophilia patients with HCV infection, even after successful clearance, especially those with significant fibrosis and/or co-morbidities. HBV infection is less common in PWH than HCV and is often detected late, when HBsAg disappears and only HBcAb is detectable. Chronic HBV infection progresses through five phases determined by viral replication and liver inflammation [22]. The risk of HBV-related HCC is lower in non-cirrhotics and guidelines recommend HCC screening in certain chronic hepatitis B (CHB) subgroups [22]. In particular, patients with inactive disease or a resolved infection are still at risk of reactivation on immunosuppressive therapy [22]. Nucleos(t)ide analogues (NUC) such as entecavir, tenofovir disoproxil or tenofovir alafenamide suppress HBV and improve liver disease, with a reduced risk of HCC [22]. All PWH should be vaccinated against hepatitis B [23]. HBV carriers are at risk for HDV "co-" or "super" infection and should be screened (anti-HDV antibodies and HDV-RNA in all positive cases) [24]. Bulevirtide, an HBV and HDV entry inhibitor, has been approved in Europe for HDV infection and can be prescribed in PWH [25].

3. Metabolic dysfunction-associated steatotic liver disease (MASLD)

Metabolic dysfunction-associated steatotic liver disease (MASLD) [26] is the most frequent liver disease worldwide. It results from the accumulation of triglycerides containing lipid droplets in hepatocytes in the absence of an excessive consumption of alcohol. MASLD prevalence is of about 30 % in the general population [27]. It is estimated that this figure will double by 2030, and that MASLD-related cirrhosis and HCC will triple by that time [28,29]. Sustained weight loss over 10 %, through lifestyle changes (diet and exercise) or bariatric surgery, can reverse inflammation and liver fibrosis [30]. MASLD increases the risk of liver disease progression in patients with chronic viral infections [31] and also affects overall health, contributing to insulin resistance, higher cardiovascular risk, and hypercoagulability [32].

Surprisingly, the first cases of MASLD in PWH were described in children [33]. Among 173 boys treated in American hemophilia centers, 25 were obese (14,7 %, 95 % CI 9.7–20.9 %), five had persistently elevated levels of alanine aminotransferase (ALT) and three had clinical and imaging criteria of MASLD. Sedentary lifestyle, overweight and obesity are frequent in PWH. A recent Chinese registry-based study reported a higher prevalence of MASLD (47.2 %) in adult PWH compared to the general population. In this study, overweight/obesity was associated with a 4.3 times higher risk of MASLD ($p < 0.001$) [34].

4. Hepatocellular carcinoma

The increasing life expectancy of people with hemophilia (PWH) presents new challenges in the management of associated cardiovascular diseases and cancers, particularly HCC due to hepatitis C and liver diseases [35]. HCC is the most common solid tumor in PWH [36]. Prevention, diagnosis and treatment of HCC in PWH do not differ from the general population [37,38]. Although antiviral treatments of HCV and HBV infections can reduce the risk of HCC, surveillance remains critical for early detection [38], especially in those with advanced fibrosis at the start of antiviral treatment (and regardless of the evolution of non-invasive fibrosis testing during therapy) and in those with comorbidities, i.e., alcohol consumption and/or MASLD [37]. Regular ultrasound examinations, often combined with alpha-fetoprotein (AFP) measurements, are recommended for screening [37,39]. Magnetic resonance imaging (MRI) is preferred to confirm suspicious nodules [37]. Liver resection is an option in some cases, although the risk of recurrence is high [37,39]. Targeted therapies and liver transplantation, with indications similar to the general population, offer promising treatment options for HCC in PWH, with liver transplantation providing cure rates comparable to the general population [40,41].

5. Liver disease staging in hemophilia

Liver biopsy, the "gold standard" for the assessment of liver histology, can be safely performed in PWH by experienced operators under substitution therapy. Trans-jugular liver biopsy (TJLB) has been recommended for these patients at high risk of bleeding [42,43]. One of the major limitations of TJLB is the small size of the liver sample, which may also be fragmented during the procedure thus rendering a correct histological interpretation difficult. In addition, the area of biopsy is not always very informative about the overall degree of liver damage, as some areas may be more affected than others [44] with up to 30 % of the samples misdiagnosed [44]. Iatrogenicity of the TJLB procedure, such as perforation, is another important concern [45].

Over the past 15 years, non-invasive methods, based on either serum biomarkers or liver elastography, have been developed to assess liver damage and liver fibrosis progression (46 and Table 1). Elastography includes Vibration-Controlled Transient Elastography (VCTE or

Table 1
Non-invasive tests for evaluating hepatic fibrosis and the parameters they incorporate.

Liver fibrosis assessment scores	Liver fibrosis biomarkers	Liver fibrosis assessment scores including specific biomarkers
AAR (AST to ALT ratio) ADAPT (age, presence of diabetes, PRO-C3, and platelet count) APRI (ALT to platelet count ratio) FIB-4 (age, AST, ALT, platelet count) FibroTest (GGT, α2 macroglobulin, total bilirubin, haptoglobin, apolipoprotein A1) Forns index (Age, cholesterol, GGT, platelet count) ViraHep C (Age, AST, alkaline phosphatase, platelet count)	Collagens IV and VI Hyaluronic acid, laminin PIIINP TIMP 1-2 MMP 1-2-9 Cytokines: IL 6, IL 10, TNF α, IFN ϒ Growth factors: TGFβ, YKL-40	Enhanced Liver Fibrosis test (hyaluronic acid, pro-collagen III and TIMP1) Fibro Meter (age, platelet count, prothrombin time, AST, urea, α2 macroglobulin, hyaluronic acid) Fibro Spect (TIMP 1, α2 macroglobulin, hyaluronic acid) Hepa Score (age, sex, bilirubin, GGT, α2 macroglobulin, hyaluronic acid)

FibroScan), 2D-shear wave elastography integrated into ultrasound devices, and more recently, magnetic resonance elastography (MRE) [46]. Fibroscan measures the degree of liver stiffness to indirectly estimate the degree of liver fibrosis [46] and is widely used to stage liver fibrosis and assess prognosis [46], including PWH [47]. Fibroscan has a high degree of reliability in assessing liver fibrosis and in diagnosing cirrhosis [46]. The Enhanced Liver Fibrosis test uses a panel of matrix turnover markers (hyaluronic acid, pro-collagen III and TIMP1) [46]. FibroMeter's patented algorithm combines age, body weight, glucose, AST, ALT, ferritin and platelet count [46]. FibroTest proprietary liver fibrosis score is based on serum alfa 2-macroglobulin, haptoglobin, apolipoprotein A1, total bilirubin and g-glutamyl-transpeptidase, corrected for age and gender [46]. Of note, haptoglobin and bilirubin levels may be altered in PWH in the event of hematoma, independently of liver fibrosis extent. Therefore, a comparison of Fibroscan and FibroTest results in PWH showed an only fair agreement [48]. Simpler indirect scores based on calculations from blood results to determine the degree of liver fibrosis include the AST-to-platelet ratio index (APRI) and the Fibrosis 4 index or FIB-4 (age, platelet count, ALT and AST levels) (Table 1) [46]. A study conducted in PWH with hepatitis C or co-infection with HIV/HCV showed that the combination of two non-invasive markers could significantly reduce the need for liver biopsy in the search for advanced hepatic fibrosis [49]. To improve the accuracy of fibrosis staging in PWH, several algorithms combining biomarkers and fibroscan have been proposed [48,49]. A similar approach using 2 or more non-invasive tests and combining biomarkers and elastography is recommended for patients with MASLD whose FIB-4 score is >1.3 [50]. The American Gastroenterological Association recommends liver biopsy for patients with MASLD for whom non-invasive test results are indeterminate or discordant [50]. Fibroscan is also an effective method for staging hepatic steatosis in MASLD. Recent advances in hepatic fat and iron measurement using quantitative chemical shift-encoded MRI have made hepatic iron and fat measurement simpler and more accessible [51].

6. Gene therapy of hemophilia and liver health

Market authorization has been granted in Europe and North America for valoctocogene roxaparvovec for hemophilia A (HA) and etranacogene dezaparvovec for hemophilia B (HB). These two gene therapies are based on viral transduction of hepatocytes. They use recombinant vectors derived from the adeno-associated virus serotype 5 (AAV5), unable to replicate. In both cases, AAV5 vectors contain a codon optimized single strand of DNA (factor [F]VIII or FIX transgene) to improve expression of the deficient protein (FVIII or FIX) with a hepatocyte specific promotor. AAV5 offers better transduction efficacy in hepatocytes, achieving approximately 15 % transduced cells compared to AAV2's 5 % in mice, resulting in higher protein levels [52]. Additionally, fewer individuals are pre-immunized against AAV5 than AAV2 [53].

6.1. Hepatocytes, targets of hemophilia GT

The liver plays a central role in metabolic processes and synthesizing most coagulation proteins, making hepatocytes ideal targets for hemophilia GT. The liver's abundant vascularization ensures rapid protein transport and effective delivery of GT vectors. Liver sinusoids, with their specialized endothelium without a basement membrane and with "fenestrae," promote exchanges between the blood and hepatocytes. These small endothelial pores allow AAV vectors to efficiently reach hepatocytes [54]. In a non-inflamed liver, hepatocytes have a low renewal rate, reducing the dilution of transduced cells. In addition, hepatocytes have the necessary machinery for the production and post-translational modifications of coagulation proteins, making them the optimal target for hemophilia GT.

6.2. Hepatic adverse events in hemophilia GT

6.2.1. Hemophilia A GT with valoctocogene roxaparvovec

The phase I/II trial, that included nine patients, evaluated the efficacy and safety of two doses of vector/genomes, respectively 4×10^{13} and 6×10^{13}/kg body weight. Moderate increases in alanine aminotransferase (ALT) were observed in 78 % of the participants during the first year, regardless of the dose [55]. In the phase III study, 134 patients with severe HA received 6×10^{13} vector/genomes/kg. As in the early studies, 86 % of these patients experienced ALT elevations, and 79.1 % of them underwent corticosteroid therapy for a median duration of 230 days (range 22–551) to manage the ALT increase. Among them, 71.8 % developed mild adverse events linked to corticosteroids such as Cushing syndrome, weight gain, insomnia or acne. The 39 patients with contraindications to corticosteroid therapy received other immunosuppressive agents (mycophenolate, tacrolimus) [56].

6.2.2. Hemophilia B GT with etranacogene dezaparvovec

The phase I/II study, which included 10 patients evaluated the efficacy and safety of two doses of 5×10^{12} and 2×10^{13} vector/genomes/kg respectively, with no side effect.

Fifty-four patients with HB and FIX levels <2 IU/dL were enrolled in the phase III trial and received 2×10^{13} vector/genomes/kg. ALT levels increased in 20 % of the patients during the first year and 17 % received corticosteroids for a median duration of 79.8 days (51–130), without any steroids adverse events [57]. The hepatotoxicity rate observed after GT for HB was 20 %, comparable to that observed in other conditions such as spinal muscular atrophy, where 23 to 34 % of patients experienced liver-associated adverse events after treatment with onasemnogene abeparvovec, a GT based on an AAV9 vector [58].

6.3. Mechanisms of liver toxicity in hemophilia GT

The mechanisms underlying the frequent occurrence of liver toxicity translating in ALT elevations are multiple and complex (Fig. 1). Although recent studies have clarified some of these mechanisms and allowed the formulation of hypotheses, the pathophysiology of this hepatic response is far from being fully understood.

The first proposed mechanism is an acquired immune response directed against the capsid of the AAV vector. A transaminitis was previously observed in clinical trials using AAV2 [59] and then AAV6 [60] as vectors of hemophilia GT. It has been clearly demonstrated that $CD8^+$ cytotoxic T-cells can detect epitopes generated by AAV capsid degradation in the hepatocytes. Capsid epitopes can then be presented by

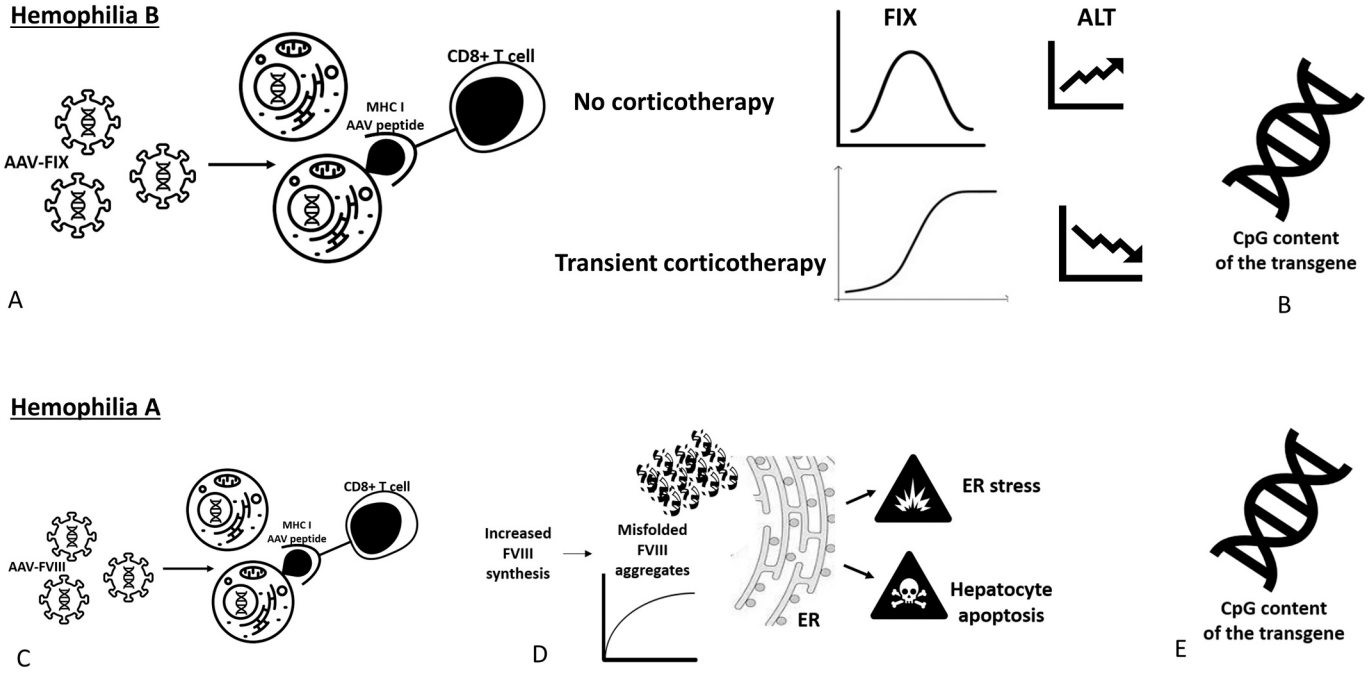

Fig. 1. Clinical studies on hemophilia A and B reveal brief, asymptomatic increases in alanine transaminase (ALT) levels, manageable with glucocorticoids. This mild toxicity is linked to viral processes and DNA damage response. Elevated ALT levels occasionally coincide with an anti-AAV capsid T-cell response, yet this correlation is inconsistent (A & C). According to Nathwani et al. [62], a potential role of non-specific innate immunity towards unmethylated CpG nucleotides present in the transgene, liable to activate Toll-receptor (TLR)-9 is another cause of hepatic toxicity (B & E). The liver's multifaceted functions make it susceptible to endoplasmic reticulum (ER) stress. Transgene expression in specific cells may overload some with factor VIII (FVIII), inducing cellular stress. ER, crucial for protein folding, faces stress from increased folding demands or accumulation of misfolded proteins, activating the unfolded protein response (UPR) and generating additional ER to mitigate stress. Overwhelming stress may lead to apoptosis. Understanding these pathways is vital for hemophilia treatment and gene therapy outcomes (D).

hepatocytes in the context of Class I major histocompatibility complex antigens, leading to their killing by AAV-specific cytotoxic T-cells. The intensity and duration of the $CD8^+$ response seems to be influenced by the AAV serotype used [61]. Corticosteroids appear to be capable to control this acquired immunity (Fig. 1A and C) [62]. Achieving ALT normalization is important to decrease the extent of hepatocyte death and maintain the expression of FVIII or IX in the transduced liver and consequently the therapeutic effect. A study performed in HA mice, who had received AAV5 GT and were systematically treated by prednisolone, showed no reduction of FVIII expression in the hepatocytes compared to

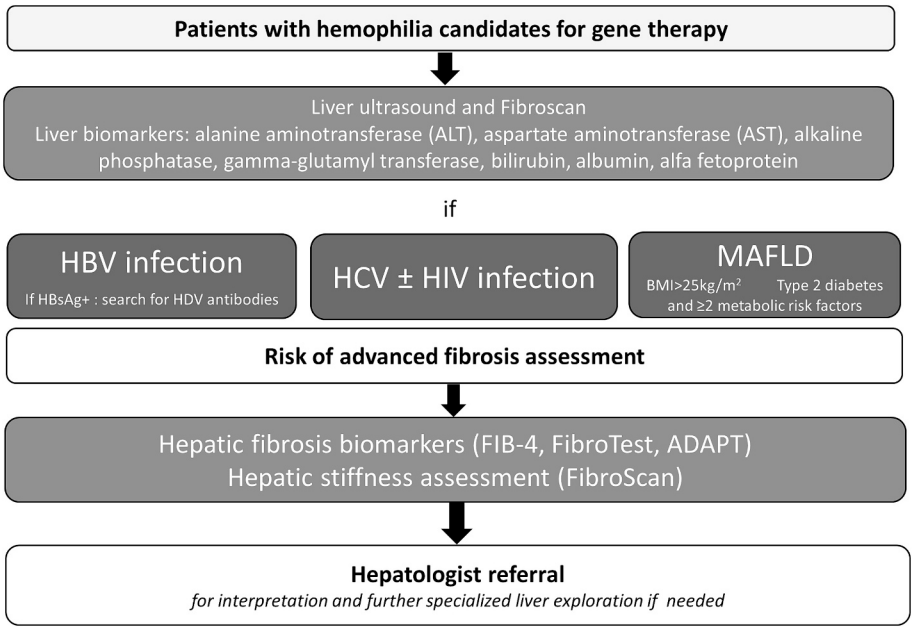

Fig. 2. Strategy for evaluating liver health in hemophilic patients eligible for gene therapy. HBV: hepatitis B virus; HCV: hepatitis C virus; HIV: human immunodeficiency virus; BMI: body mass index; MAFLD: Metabolic dysfunction-associated fatty liver disease; Metabolic risk factors: waist circumference $\geq$ 102/88 cm; blood pressure $\geq$ 135/80 mm Hg or specific drug treatment; plasma HDL cholesterol <1.0 mmol/L; high sensitivity C reactive protein (hs-CRP) >2 mg/L; insulin resistance score $\geq$ 2.5; prediabetes (fasting glucose levels >5.6–6.9 mmol/L) or HbA1c 5.7 to 6.4 %.

mice given a placebo. However, no significant ALT elevation was observed in mice treated with AAV5-hFVIII-SQ, regardless of prednisolone administration or vector dose [63].

Other preclinical studies have highlighted the potential role of non-antigen specific innate immunity towards the unmethylated CpG nucleotides present in the transgene mediated by Toll-receptor (TLR)-9 activation (Fig. 1B and E) [64].

A clinical trial of HB GT (BAX 335; AskBio009, AAV8.sc-TTR-FIXR338Lopt) has shown a loss of FIX expression between 5- and 11-weeks post-injection, without any protective effect of corticotherapy. The authors proposed that the CpG deoxy oligonucleotides introduced in the transgene as part of the codons optimization could be responsible for a strong activation of innate immunity leading to hepatocyte destruction [65].

It is surprising to observe that 85 % of patients with HA and only 20 % with HB develop transaminitis after GT (Fig. 2). This important difference is likely due to complex mechanisms, including the choice of target cells. Indeed, hepatocytes are the natural site of FIX production, whereas FVIII is produced by sinusoidal endothelial cells [66]. Another explanation could be the molecular differences between FVIII and FIX. They are very different in size (330 kDa vs. 57 kDa) as well as in the length of their genes. Complementary DNA (cDNA) of FVIII is 9 kilobases (kb)-long while the size of FIX cDNA is only 2.8 kb. The latter is therefore much easier to introduce as a transgene into AAV vectors, which cannot accommodate >5 kb. The limited capacity of AAVs makes impossible to insert the whole sequence of FVIII in the vector and all HA GT strategies use B domain-deleted FVIII (BDD-FVIII), which ranges in size between 4.4 and 4.7 kb. Both wild type FVIII and BDD-FVIII are difficult to be secreted by the cells that have been transduced.

In vivo, in physiological conditions, endothelial cells cleave FVIII signal peptide in the endoplasmic reticulum (ER), and more proteolytic cleavages occur during transition towards the Golgi apparatus. This allows FVIII to acquire its heterodimeric structure with a heavy and a light chain.

When synthesized in hepatocytes, FVIII is often improperly folded, which leads to an accumulation of ill-folded proteins that cannot be secreted and form aggregates within the ER (Fig. 1D). This inefficient intra-cellular traffic from the ER to the Golgi apparatus triggers a cellular stress response [67]. Increasing the level of protein expression would not necessarily lead to higher blood levels of FVIII, as the challenge lies in secretion rather than synthesis. A pre-clinical study in HA mice has also shown some impact of the promoter chosen for the FVIII transgene [68]. A strong promoter may indeed induce a substantial synthesis of FVIII but would result in more ER stress compared to a weaker promoter. However, the latter may facilitate more efficient and potentially sufficient production, especially when considering a high proportion of transduced hepatocytes.

In hemophilia GT, the target cell for FVIII or FIX synthesis and the cell that suffers the toxic side effect is the same, i.e., the hepatocyte. As previously reported in preclinical studies, elevated ALT following GT may be an important biomarker of potential hepatotoxicity. Vigilant monitoring and timely intervention are essential to maintain the integrity of transduced hepatocytes responsible for FVIII or FIX expression. Both valoctocogene roxaparvovec and etranacogene dezaparvovec product characteristics indicates that during the first year after hemophilia GT, ALT and factor VIII or IX activity levels should be monitored and corticosteroid treatment should be instituted in response to ALT elevations as needed, to control hepatic reactions and prevent or mitigate a potential reduction in transgene [69,70]. The clinical difficulty is in results interpretation. Transduction itself can result in an elevation of transaminases, as observed in other therapies targeting hepatocytes, like small interfering RNAs (siRNA) that downregulate antithrombin expression by hepatocytes, where hepatoxicity may be related to activation of the innate immunity by the siRNA [71]. At what point should the increase in ALT be regarded as the anticipated liver response, and beyond which threshold should it be construed as hepatocyte destruction? How can the latter be differentiated from an intra-individual fluctuation, or an artefactual increase of ALT due to sustained muscular effort (where AST is higher than ALT and is associated with an elevated CPK), or a side effect of concomitant exposure to hepatotoxic drugs, alcohol, dietary supplements or analgesics, or even a pre-existing underlying liver disease such as MASLD or rare liver disorders?

Therefore, a thorough evaluation of liver function prior to GT is mandatory to improve the chances of successful GT and manage successfully liver toxicity.

6.4. Liver function evaluation before GT

Caution is advised due to liver cell problems that may affect hemophilia GT efficacy. Valoctocogene roxaparvovec is unsuitable for patients with acute or uncontrolled chronic infections, significant fibrosis, cirrhosis, other liver diseases, or abnormal liver function tests. Screening for liver cancer is recommended before prescribing this therapy to ensure patient safety [69]. GT Etranacogene dezaparvovec requires a thorough evaluation of liver health before administration, with consultation with a hepatologist recommended if liver abnormalities persist [70]. All candidates for hemophilia GT should therefore undergo a detailed liver health screening in collaboration with a hepatologist (Fig. 2). The following laboratory parameters were typically included in the pre-treatment evaluation of candidate patients in the Phase 3 trials: ALT, aspartate aminotransferase (AST), alkaline phosphatase, gamma-glutamyltransferase (GGT), bilirubin, albumin, and AFP. Baseline AST/ALT levels may need to be assessed two- or three-times during screening due to spontaneous fluctuations. Patients with pre-existing liver disease, immune disorders, or untreated viral hepatitis may require more extensive evaluation. Viral load, measured by HBV-DNA or HCV-RNA, will determine the need for preemptive antiviral therapy to avoid contraindications to immunosuppression after GT. In NUC-treated CHB patients, treatment should be optimized to ensure viral suppression and normal liver function tests before starting GT. In all HBsAg-positive patients, antibodies to HDV should be investigated. Patient with CHC should be treated with DAA before GT. In HIV infection, it is essential to monitor the viral load and CD4+ T-cell count to ensure that they are undetectable or <20 copies and $>0.2 \times 10^9$/L, respectively.

A recent study of the prevalence and risk factors for HCC in over 3700 PWH and healthy controls showed that among HCCs, hemophiliacs were younger and more likely to be HIV+, both $p < 0.001$, but less likely to be alcoholic ($p = 0.018$) or hyperlipidemic ($p = 0.008$) compared with normal controls. Despite the lack of specific data to predict individual risk for each hemophilia patient, multivariable regression analysis identified MASLD as a significant risk factor for HCC in PWH, with an odds ratio of 21.6 [72]. Improving the efficacy and safety profile of hemophilia GT requires careful consideration of hepatotoxicity, particularly emphasizing the importance of screening for MASLD during pretreatment evaluation. The need for liver exploration extends to a broader spectrum of rare liver diseases, including autoimmune liver diseases [73].

Abdominal ultrasound is needed to investigate signs of liver disease and/or portal hypertension, and Fibroscan® for non-invasive assessment of liver fibrosis. In most cases, Fibroscan® < 10 kPa rules out severe liver fibrosis, but this test must be interpreted in the context of possible factors that may alter liver elasticity independently of fibrosis, such as inflammation, cholestasis, and hepatic congestion. Relying solely on a Fibroscan® threshold as the eligibility criterion for GT is not recommended. Expert guidance from a hepatologist is essential in making informed decisions regarding eligibility. A FIB-4 score, platelet count and albumin level may/should be obtained prior to consultation with the hepatologist, who may request additional second-line non-invasive tests for fibrosis on a case-by-case basis. The potential liver toxicity of the medications currently used by the patient can be assessed by accessing www.livertox.nih.gov.

6.5. Liver function monitoring during the first year after GT

PWH who have undergone GT require extensive clinical and laboratory monitoring during the first year to assess transgene expression levels and potential treatment-related hepatotoxicity. Regular blood tests should include a complete blood count (CBC), FVIII (by chromogenic method) or FIX (by coagulometric method) activity assays, and liver function tests (AST, ALT, bilirubin, alkaline phosphatases, GGT). The scheduling of FVIII or FIX and liver function tests in the first six months varies based on the specific risk of hepatotoxicity associated with each product. For valoctocogene roxaparvovec in HA, it is advisable to perform weekly evaluations for the first 6 months, then every 2 to 4 weeks from month 6 to 12 [69]. Regarding the etranacogene dezaparvovec in HB, the lower risk of hepatotoxicity allows a less stringent follow-up, with weekly assessments for the first 3 months and then every 3 months [70].

Any ALT elevation after GT must be confirmed after 48 h for early detection of immuno-mediated flares that could irreversibly affect transgene expression [74]. Patients who have received GT must avoid alcohol consumption for at least 6 months after injection. The gamma GT assay can be useful to interpret ALT results and CPK levels could help identifying increases related to intense physical activity.

Finally, for patients with hemophilic arthropathy responsible for chronic pain, modification of analgesics can be necessary, limiting opioids to short periods, using cyclooxygenase-2 inhibitors (celecoxib) as anti-inflammatory drugs and limiting high doses (>3 g/d) of paracetamol [75].

6.6. Diagnosis and management of hepatotoxicity related to GT

Close monitoring of ALT is crucial to prevent loss of transgene expression in cases of AAV-induced liver toxicity. While corticosteroids are typically the first-line treatment unless contraindicated, it's important to consider other factors that can elevate ALT levels. These include acute viral hepatitis, alcohol consumption, hepatotoxicity from concomitant medications, or intense physical activity, all of which can cause moderate and transient ALT elevations [74]. In GT trials, hepatotoxicity is often defined as a 30–50 % increase in ALT from the patient's lowest recorded level, even if ALT remains within the normal range. If an increase of >1.5 times the baseline value is observed and confirmed in a second blood sample within 48–72 h in the same laboratory, initiating immunosuppression should be discussed in a multidisciplinary meeting involving hepatologists. Corticosteroids are usually initiated at 1 mg/kg/day for two weeks and tapered gradually as ALT levels normalize. Tapering should be slow, e.g., decreasing by 10 mg every two weeks, due to the high risk of relapse with rapid tapering. If corticosteroids are contraindicated, other immunosuppressive drugs such as azathioprine, mycophenolate mofetil, or tacrolimus can be considered [76,77].

6.7. Long term hepatic follow-up after GT

Health authorities recommend a 15-year follow-up for PWH after GT, but lifelong surveillance with consistent data collection is essential to improve our understanding of its long-term benefits and risks. National databases, along with the international registry of the World Federation of Hemophilia [78,79], are crucial for comprehensive long-term data collection.

The primary long-term risk is liver cancer, influenced by several factors:

- AAV vectors: AAVs exhibit episomal organization within transduced hepatocytes' nuclei, with low integration into the human genome. In rodent models, high doses of AAV vectors with strong promoters and neonatal age may increase liver tumor risk due to higher hepatocyte proliferation rates [80]. Human GT protocols have been optimized accordingly. Notably, in a preclinical study in hemophilic dogs treated with AAV-FVIII GT over a decade ago, no cases of hepatocarcinoma were reported [81]. However, the finding, in a very small proportion of HCCs, of AAV2 clonal integrations in genes involved in hepatocarcinogenesis suggests that a low risk may remain [82,83]. More than 320 PWH have undergone GT with follow-up ranging from 0.5 to 15 years. In 2020, a case of HCC was reported in a patient from the HOPE-B trial who received etranacogene dezaparvovec [84]. The patient, over 65 years old, had a history of hepatitis B, cured hepatitis C, and evidence of MASLD on liver biopsy. Molecular analysis of the tumor revealed chromosomal abnormalities and HCC-associated mutations but no association with AAV integration [84].
- ER stress: The FVIII transgene, which results in misfolded FVIII molecules that accumulate in the ER, might lead to transformation of hepatocytes into HCC [67].
- Patient hepatic history: Even if viral hepatitis has been successfully treated before GT, the risk of HCC remains [85], especially considering its prevalence in this population [36]. Long-term follow-up with liver ultrasound and AFP assay is recommended. MASLD/NASH also increases HCC risk [86]. Obese patients with MASLD remain eligible for GT but require regular monitoring for HCC risk. A recent preclinical study in mice with hemophilia A showed that those on a high-fat diet after GT developed HCC, highlighting the risks associated with MASLD and ER stress [87]. This emphasizes the importance of vigilant patient monitoring and adopting a healthy lifestyle post-GT. Emerging risk factors for HCC after GT, derived from animal studies, await validation in humans. Due to the early stage of GT, evidence-based recommendations for HCC screening are lacking. While AAV vectors may ultimately prove unrelated to HCC risk, we advocate a cautious approach with ongoing surveillance until further clarity is achieved [86].

7. Insights gained from investigating liver toxicity in other AAV-based gene therapies

Onasemnogene abeparvovec GT for spinal muscular atrophy, using an AAV9 vector, is associated with hepatotoxicity [88]. Intravenous administration of AAV9, though effective for gene transfer, requires remarkably high doses to target the central nervous system (CNS), often exceeding 1×10^{14} vg/kg. This high dose requirement is due to the predominant distribution of AAV genomes in organs outside the CNS, particularly the liver. Consequently, in a minority of patients, this significant biodistribution to non-CNS organs may lead to clinically relevant adverse effects such as hepatotoxicity, thrombocytopenia with or without thrombotic microangiopathy (TMA), or cardiac toxicity [89].

Sixty percent of infants had abnormal transaminase and bilirubin levels before onasemnogene abeparvovec GT. 90 % experienced two distinct peaks of ALT elevation, occurring one week and one-month post-GT. Liver biopsies in two children with ALT levels exceeding 40 times the upper limit of normal and concurrent bilirubin elevation revealed predominantly CD8+ lymphocytic infiltrates and evidence of liver fibrosis. Systematic preventive corticosteroid treatment is under consideration for those undergoing onasemnogene abeparvovec GT due to these findings, emphasizing the need for continued research and proactive measures to address potential hepatotoxicity associated with AAV-based GT. Further liver biopsy studies post-GT may shed light on some of the uncertainties surrounding hemophilia GT.

A recent sub-study within the GENEr8–1/3 trials, evaluating 6.10^{13}vg/kg valoctocogene roxaparvovec in adults with severe HA, analyzed liver biopsies from 12 patients. The results showed low FVIII RNA transcription rather than loss of the full-length transgene, suggesting epigenetic silencing affects FVIII expression levels and activity, potentially leading to declining levels over time. These biopsy studies may provide additional understanding of the mechanisms underlying transaminitis and its consequences [90,91].

8. Conclusion

Hematologists in hemophilia centers and hepatologists have a long history of collaboration, particularly in managing hepatitis B and C. This partnership is expected to deepen with the introduction of GT. Their collaboration is essential for several aspects: interpreting pre-GT liver tests during eligibility assessment, diagnosing and managing post-GT liver toxicity, screening for silent hepatitis B before prescribing corticosteroids, considering liver biopsy in complex cases, and preventing viral hepatitis reactivation during immunosuppression, especially in those with a history of hepatitis B. This multidisciplinary approach aims to mitigate liver-related risks associated with hemophilia GT. It requires a structured organization with frequent hepatologist involvement in patient management. At the same time, ongoing data collection and research are essential to understand the mechanisms of hepatic toxicity, improve early diagnosis, and establish optimal management strategies.

CRediT authorship contribution statement

Yesim Dargaud: Writing – review & editing, Writing – original draft, Conceptualization. **Massimo Levrero:** Writing – review & editing, Writing – original draft. **François Bailly:** Writing – review & editing. **Anne Lienhart:** Writing – review & editing. **Fabien Zoulim:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

Data sharing not applicable – no new data generated.

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