

# The Evolution of Hemophilia Care: Clinical and Laboratory Advances, Opportunities, and Challenges

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## Abstract

Hemophilia A (HA) and B (HB) are X-linked bleeding disorders caused by mutations in the F8 or F9 gene that result in the absence, or reduced activity, of the corresponding clotting factor. The severity of bleeding and related complications is proportional to the amount of residual circulating functional factor. The development of a safe and effective hemophilia treatment lasted several decades and has been mainly based on clotting factor replacement. Advances in the engineering and manufacturing of clotting concentrates have led to the widespread availability of extended half-life products that reduced the number of intravenous infusions needed to achieve adequate trough levels. The recent development of new nonfactor replacement treatments and biotechnology techniques has offered therapeutic alternatives for hemophilia patients with and without inhibitors. These are characterized by an easier route of administration, low immunogenicity, and, regarding gene therapy and cell-based treatments, potential long-term protection from bleeding after a single treatment course. In this review, we analyze recent progresses in the management of hemophilia and discuss opportunities and challenges.

## Keywords

- ▶ hemophilia A/B
- ▶ gene therapy
- ▶ factor VIII
- ▶ factor IX

## Introduction

Hemophilia A (HA) and B (HB) are X-linked bleeding disorders caused by mutations in the F8 or F9 gene that result in the absence, or reduced activity, of the corresponding clotting factor.<sup>1</sup> The severity of clinical manifestations is proportional to the amount of residual circulating functional factor. In severe cases (clotting factor level <0.01 IU/mL), spontaneous bleeds are an integral part of the clinical picture. These are typically hemarthrosis (intra-articular bleeding). Muscle bleeding, soft tissue hemorrhage, and intracranial hemor-

rhages (ICHs) are also frequently described.<sup>2</sup> Repeated hemarthroses lead to a hypertrophic synovitis characterized by an increased production of soluble mediators of inflammation with progressive cartilage degradation and bone damage, chronic pain, and irreversible joint function impairment.<sup>3</sup> On the other side of the severity spectrum, patients with higher levels of circulating factors and moderate or mild manifestations of disease may experience bleeding only after traumatic events or surgical procedures.<sup>4</sup> Moderate hemophilia is defined for FVIII:C levels of 0.01 to <0.05 IU/mL. Mild HA is characterized by either FVIII:C levels

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of  $<0.4$  IU/mL or FVIII:C  $>0.4$  IU/mL plus evidence of a listed F8 DNA-mutation associated with HA and FVIII:C  $<0.4$  IU/mL or a family member with the same DNA change and FVIII:C  $<0.4$  IU/mL.<sup>4</sup>

The development of a safe and effective hemophilia treatment required several decades of efforts and has been mainly based on replacement of the missing clotting factor. Barriers to adequate therapy remain and limit the achievement of an adequate bleeding control and prevention of joint deterioration, such as difficult venous access—particularly in infants—the high frequency of intravenous treatment, and the development of an immune response toward the therapeutically administered clotting factor. The latter, neutralizing antibodies to exogenous factor denominated “inhibitors,” is a major issue in hemophilia treatment that precludes the possibility of an appropriate prophylactic treatment to these patients.

Advances in the engineering and manufacturing of clotting concentrates have led to the widespread availability of extended half-life (EHL) products that reduced the number of intravenous infusions needed to achieve adequate trough levels. The recent development of new non factor replacement treatments and biotechnology advances has offered therapeutic alternatives for hemophilia patients with and without inhibitors, characterized by an easier route of administration, low immunogenicity, and, regarding gene therapy and cell-based treatments, potential long-term protection from bleeding after a single treatment course.

In this review, we analyze recent progresses in the management of hemophilia and discuss opportunities and challenges.

### The Evolution of Factor Replacement Therapy and Its Impact on Life Expectancy

The treatment of hemophilia has been based on coagulation factor replacement for the past 60 years. In the 1960s, novel plasma fractionation techniques were instrumental for the extraction of high-concentration factor VIII (FVIII) and von Willebrand factor from the precipitate of frozen plasma. Cryoprecipitates became the first effective therapy of hemophilia-related bleeding.<sup>5</sup> The industrial manufacturing of plasma-derived highly enriched FVIII and factor IX (FIX) concentrates broadened the availability of supplies. This translated into an earlier control of hemorrhages and the opportunity to opt for home treatment,<sup>6</sup> but also favored the transition from an “on demand” treatment to routine bleeding prophylaxis.<sup>1</sup> The first national programs of specialized hemophilia treatment centers were launched with the objective to provide a specialized, comprehensive care.

In these first years, life expectancy of hemophilia patients dramatically increased (–Table 1). In the late 1950s, half of the patients with hemophilia (PwH) would have died by the age of 19 years, whereas the median life expectancy reached approximately 50 years in Northern Europe and America in the early 1980s.<sup>7,8</sup> This was accompanied by an improvement in quality of life (QoL), particularly among severe patients,<sup>9–11</sup> and a reduction of the burden of hemophilia arthropathy.<sup>12</sup> These positive trends were abruptly interrupted in the 1980s: the human immunodeficiency virus (HIV) and non-A hepatitis viruses spread among the hemophilic population, as clotting

factors concentrates were produced by pooling the plasma from thousands blood donors.<sup>13</sup> Routine viral testing of the blood donors would have been introduced only in the year 1985. Novel purification methods and viral inactivation/removal techniques for the production of plasma-derived concentrates were progressively implemented to minimize the risk of new infections.<sup>14,15</sup> In the United States, the median age of death fell to 35 years in the years 1993 to 1995 at the peak of the HIV epidemic with acquired immune deficiency syndrome (AIDS) recorded as the immediate or underlying cause in more than 60% of deaths in PwH (–Table 1).<sup>16,17</sup>

F8 was cloned in 1984,<sup>18</sup> paving the way to the production of recombinant FVIII-concentrates, which consisted of three generations of products: (1) animal-derived proteins with human serum albumin added, (2) human-derived proteins without albumin, and (3) manufactured FVIII only.<sup>19</sup> The last two generations included concentrates lacking the FVIII B-domain, which appeared to be unnecessary for FVIII-coagulant activity, but rather involved in intracellular trafficking, secretion, and possibly implications for immunogenicity.<sup>20</sup>

The availability of safe and effective replacement therapy with factor concentrates substantially improved the care of hemophilia patients. However, the formation of inhibitory alloantibodies against the infused FVIII and FIX is a major complication of the treatment with clotting factor concentrates. Inhibitors develop in 20 to 40% and 3 to 10% of patients affected by severe HA and HB, respectively, mostly within the first 50, but also up to 100 to 500 exposure days.<sup>21,22</sup> A lower amount of patients affected by mild or moderate HA, namely 3 to 13%, could also develop an immune response to the infused clotting factor.

The presence of inhibitors causes ineffectiveness of the treatment with a consequent increase in morbidity and mortality.<sup>23,24</sup> Agents bypassing the inhibitors activity, such as activated prothrombin complex concentrate (aPCC) and recombinant activated factor VII (rFVIIa), have been available since the 1970s to 1980s, albeit their efficacy is lower than that of FVIII concentrates,<sup>25</sup> and are characterized by higher costs and treatment burden.<sup>26</sup>

The modern approach to factor replacement therapy consisted of (1) “on demand” treatment to treat acute bleeding; (2) short-term prophylaxis before surgical or invasive procedures; (3) primary long-term prophylaxis starting before the age of 2 years and after no more than one joint bleed, given at a fixed dose mostly two to three times per week; (4) routine or intermittent treatment in the context of a secondary or tertiary prophylaxis to prevent recurrent bleeding events and their complications.<sup>27</sup> Evidence from clinical trials and a recent systematic review illustrated the reduction of bleeding rate if a secondary<sup>28–31</sup> or primary prophylaxis<sup>32</sup> was in place, as compared with an on-demand treatment.<sup>33</sup> The benefits are larger if the prophylaxis is started prior to development of target joints damage<sup>34</sup> and are particularly pronounced in terms of ICH reduction.<sup>35</sup>

The increased trend in life expectancy and the improved QoL observed over the past 20 years among PwH is due, at least in part, to the high-quality factor concentrates and progress in the management of blood-borne viral infections,

**Table 1** Time trends in life expectancy and underlying causes of death in persons with hemophilia A and B

Country	Years	Persons with hemophilia	Median life expectancy or median age at death (y)	Main causes of death
Sweden <sup>7,8</sup>	1831–1980	Severe hemophilia	Median life expectancy 1831–1920: 11 y 1921–1940: 23 y 1941–1960: 36 y 1961–1980: 57	Hemorrhage (ICH 33%)
Netherlands <sup>37</sup>	1973–1992	Severe hemophilia	Median life expectancy 1973–1986: 63 y 1986–1992: 61 y	1973–1986: cancer 35% 1986–1992: AIDS 27%
United States <sup>16</sup>	1993–1995	PwH, HIV–	Median life expectancy at birth 39 y (PwH), 64 y (HIV–) Median age at death 35 y (PwH), 67 y (HIV–)	AIDS 64% (53% final cause, 12% underlying cause)
United States <sup>17</sup>	1979–1998	Hemophilia A	Median age at death 1979–1982: 50.6 y 1987–1990: 44 y 1995–1998: 50.8 y	1979–1982: circulatory 43% 1987–1998: HIV 57–47%
Netherlands <sup>38</sup>	1972–2001	Severe hemophilia	Median life expectancy 1972–1985: 63 y 1985–1992: 61 y 1992–2001: 59 y	1973–1986: hemorrhage 47% 1986–1992: AIDS 27% 1992–2001: AIDS 26%, hepatitis C 22%
United Kingdom <sup>39</sup>	1977–1999	Severe, moderate, or mild hemophilia	Median life expectancy 1999: 63 y (severe), 75 y (moderate or mild)	Hemorrhage (particularly ICH), and liver disease
Italy <sup>36</sup>	1990–2007	Severe hemophilia A, severe hemophilia B	Median age at death 39 y (HA), 35 y (HB) Median life expectancy 1995: 59.2 y (HA), 40.3 y (HB) 2005: 64.5 y (HA), 66.3 y (HB)	1980–1999: AIDS 56–60% 2000–2007: hemorrhage 27%
United States <sup>42</sup>	2007	PwH	Median age at death 68 y	Sepsis 38%
Sweden <sup>43</sup>	1968–2009	PwH	Median age at death 1981–1990: 46 y 1991–2000: 41 y 2001–2008: 56 y	Cancer 22 and 24% (total and HIV–) AIDS 8 and 31% (total and severe)
Korea <sup>45</sup>	1991–2012	PwH	Median life expectancy 2010: 69 y	Hemorrhage 52.6% (ICH 36.5%)
Taiwan <sup>40</sup>	1997–2009	PwH	Median age at death 1997–2004: 36.3 y 2005–2009: 51.8 y	Not available
United States <sup>44</sup>	1998–2011	PwH with or without inhibitors	Median age at death 39.5 y (with inhibitors) 40.4 y (without inhibitors)	Hemophilia-related 42% (inhibitors) Liver disease-related 32% (no inhibitors)
Netherlands <sup>41</sup>	1980–2011	Nonsevere hemophilia A	Median age at death 1980–1989: 32 y 1990–1999: 59 y 2000–2010: 69 y	1980–1989: ICH 20% 1990–1999: AIDS 17% 2000–2010: cancer 32%
Iran <sup>46</sup>	1975–2015	Hemophilia A, hemophilia B	Median age at death 17.2 y, 33.1 y	Hemorrhage 65–25%

Abbreviations: AIDS, acquired immune deficiency syndrome; HA, hemophilia A; HB, hemophilia B; HIV, human immunodeficiency virus; ICH, intracerebral hemorrhage; PwH, person with hemophilia.

notably HIV and hepatitis (–Table 1).<sup>7,14,16,17,36–46</sup> However, the mortality due to ICH was not remarkably affected, especially in patients without inhibitors and in moderate/mild hemophilia patients.<sup>39,47,48</sup>

Recombinant FVIII and FIX concentrates with EHL have been developed based on novel techniques, such as (glycol)

PEGylation, Fc fusion, or sequence modification. These products are characterized by higher plasma levels and a reduced frequency of administration.<sup>49</sup> Due to a wider access to high-quality prophylaxis and a comprehensive care of associated complications, PwH are now able to achieve previously unattainable life expectancy, close to that of general population in

many countries. Treatment goals have evolved from simply preventing early death to decreasing spontaneous and subclinical bleeding, prevent disability and late-onset diseases (e.g., cardiovascular diseases, cancer), and improve QoL.

### Gaps of Knowledge

Although pharmacokinetic algorithms to individualize primary and secondary prophylaxis are in place<sup>50</sup> and novel products characterized by a prolonged half-life became available, hemophilic arthropathy is not adequately prevented over lifetime; similarly, the risk of ICH among PwH exceeds that of the general nonhemophilic population.<sup>51–53</sup> A conservative prophylactic approach based on the historical threshold of FVIII  $\geq 1$  IU/dL derived from prior studies on cryoprecipitates<sup>54,55</sup> has been recently questioned. A higher threshold of FVIII (e.g., 15% or higher) has been proposed and may be reasonable for selected patients.<sup>56</sup> Not only the dosing, but also the timing of treatment initiation may be critical. An earlier prophylaxis, e.g., starting at the time of diagnosis (at birth for severe hemophilia), is crucial for maintaining healthy joints and preventing ICH during the first months of life.<sup>33</sup>

A stratification strategy has the goal to predict the occurrence of inhibitory alloantibodies and the outcome of immunotolerance induction (ITI), the treatment of choice to eradicate the inhibitors. Several genetic (ethnicity, F8-mutations, major histocompatibility complex) and acquired (number of FVIII-exposure days, age at first exposure to FVIII concentrate, type of concentrate) factors influence the development and course of inhibitors,<sup>21,57,58</sup> including potential differences in immunogenicity across factor concentrates in previously untreated patients.<sup>59,60</sup>

The rising of late-onset diseases (cardiovascular, metabolic and renal disorders, cancer) following the improved life expectancy in the hemophilia population represents nowa-

days a new challenge for patients and physicians, whereas the reduction in hepatitis C virus (HCV) and HIV infections and available curative therapies for HCV infection lead to a new generation of HIV- and HCV-free patients. This will likely affect future trends in morbidity and mortality.

### Novel Therapies for Hemophilia

New treatments have been designed that are less burdensome for the patients and more effective, irrespective of the presence of inhibitors. These nonfactor therapeutic products are long-acting and can be administered subcutaneously. Their mechanisms aim at rebalancing the hemostatic defect by targeting some of the natural anticoagulant pathways that regulate hemostasis (e.g., fitusiran, concizumab), by enhancing coagulation (emicizumab), or providing a long-term solution to factor deficiency (gene therapy).

#### Emicizumab

Emicizumab is a humanized bispecific monoclonal antibody binding FIXa and FX and thus, as a cofactor mimetic, replacing the function of the missing FVIIIa and allowing for the formation of a functional tenase complex.<sup>61</sup> The relatively weak binding affinities reduce the interference within the coagulation cascade and permits the release of FXa from the complex and participate in the downstream reactions.<sup>49</sup> Emicizumab does not have any homology with FVIII and therefore is not inhibited by the presence of neutralizing anti-FVIII antibodies. Additionally, being a monoclonal antibody has the advantage of subcutaneous administration and long half-life. A phase 3, multicenter trial was conducted to assess once-weekly subcutaneous emicizumab prophylaxis in patients with HA and FVIII inhibitors (HAVEN 1; [Table 2](#)).<sup>62</sup> Subjects randomized to emicizumab (treated weekly with 3 mg/kg as loading doses for 4 weeks and then followed by weekly injections of 1.5 mg/kg)

**Table 2** Novel treatment options for the treatment of hemophilia: features and trials data

Drug	Study	Regimen	ABR (median)	Zero bleeding rates
Emicizumab	HAVEN 1 <sup>62</sup>	1.5 mg/kg per wk	2.9	63%
		No prophylaxis	23.3	6%
	HAVEN 2 <sup>63</sup>	1.5 mg/kg per wk	0.3	77%
		3.0 mg/kg every 2wk	0.2	90%
		6 mg/kg every 4wk	2.2	60%
	HAVEN 3 <sup>64</sup>	1.5 mg/kg per wk	1.5	50%
		3.0 mg/kg every 2wk	1.3	40%
		No prophylaxis	38.2	0%
HAVEN 4	6 mg/kg every 4wk	4.5		
Fitusiran	OLE (presented at ISTH 2017)	50/80 mg once a month	0	22/33
Concizumab	explorer4 <sup>72</sup>	0.15 mg/kg with sequential dose escalation (0.20, 0.25) in case of 3 spontaneous bleedings or more	HAwl: 3	
	explorer5 <sup>72</sup>		HBwl: 5.9	
			HA: 4.5	

Abbreviations: ABR, annualized bleeding rate; HAwl, hemophilia A patients with inhibitor; HBwl, hemophilia B patients with inhibitor; ISTH, International Society on Thrombosis and Haemostasis.

were compared with a control group treated with on-demand bypassing agents, showing a difference in the annualized bleeding rate (ABR, 87%) in favor of emicizumab prophylaxis. In the HAVEN 2 trial, pediatric patients with severe HA with inhibitors were treated with emicizumab. Prophylaxis with once-weekly subcutaneous emicizumab resulted in a very low bleeding rate in children with HA and FVIII inhibitors. The authors reported a lower bleeding rate with once-weekly emicizumab compared with prior standard prophylaxis, and the majority of participants (77%) had no treated bleeding events.<sup>63</sup>

The efficacy of prophylaxis with emicizumab in HA without inhibitors has been evaluated in the HAVEN 3 trial using weekly or biweekly dosing. The results demonstrated a 68% reduction in treated bleeds compared with prior FVIII prophylaxis.<sup>64</sup> This finding supports the concept that steady-state hemostatic correction with nonfactor therapy can provide a protection possibly superior to the standard prophylaxis, which is associated with peaks and troughs of factor activity that can be more or less pronounced depending on frequency and dose of the treatment.

### Fitusiran

Fitusiran is a recently developed small-interfering RNA (siRNA) that acts by targeting and binding antithrombin (AT) messenger RNA, altering AT gene translation and blocking protein synthesis. AT can inactivate FXa and thrombin, and silencing AT causes a significant hypercoagulable state.<sup>65</sup> Because of the central role of thrombin and FXa in the physiological development and stability of clots, strategies that target AT are particularly attractive. The reduction of circulating AT can improve thrombin generation and has the potential to attenuate the bleeding symptoms.<sup>66</sup> In fact, in hemophilic mice, the targeting of AT via siRNA resulted in protection from bleeding and prolonged the lifespan of the animals. The same biotechnological approach, acting on coagulation gene transcription via siRNA, has been shown to be effective in vivo in several contexts.<sup>67</sup> A phase 1 dose-escalation study was conducted in healthy volunteers and 25 hemophilic patients without inhibitors, showing that the subcutaneous administration of fitusiran could be a promising approach.<sup>68</sup> These studies also showed the potential for monthly dosing, and the applicability to patients with HA and HB with and without inhibitors together with a low volume was used for the subcutaneous administration. Doses of 50 and 80 mg once a month in 14 inhibitor patients and 19 noninhibitor patients were tested.<sup>69</sup> The interim data analysis at a median of 13 months of treatment that was presented at the 2017 International Society on Thrombosis and Haemostasis congress demonstrated a significant decrease in AT levels. Of note, in treated patients with inhibitors, the median ABR was 0 (38 before the study), with 22/33 of patients reporting no spontaneous bleeds. Breakthrough bleeds could be successfully controlled with factor replacement therapy or bypassing agents. Phase 3 trials enrolling severe HA or HB patients, with or without inhibitors, pediatrics, and adults to further assess the safety and efficacy of fitusiran are

ongoing. A fatal severe adverse event, a cerebral vein thrombosis, occurred in a patient initially misdiagnosed as a cerebral bleeding and treated with a FVIII concentrate. After a temporary trial suspension and the development of risk-mitigation strategies to guide therapy with FVIII or bypassing agents when treating breakthrough bleeds, these trials have been reopened.<sup>70</sup>

### Anti-TFPI Antibodies

Tissue factor pathway inhibitor (TFPI) downregulates the initiation of coagulation and consists of three Kunitz-type protease inhibitor domains. It mediates the inhibition of factor VIIa/tissue factor/factor Xa (FVIIa/TF/FXa), thus inhibiting the extrinsic pathway. The K1 domain can inhibit FVIIa, the K2 domain can inhibit FXa, and the K3 domain binds protein S (PS). The rationale of targeting TFPI is that blocking TFPI inhibition may restore TF/FVIIa-mediated FXa generation to enhance in vivo hemostasis significantly. Experiments conducted in hemophilia animal models have demonstrated that the inhibition of TFPI can correct the bleeding tendency and reduce blood loss with an effect comparable to rFVIIa.<sup>71</sup>

Several clinical trials with monoclonal antibodies targeting TFPI have been started in hemophilia patients with and without inhibitors. As for emicizumab and fitusiran, also new therapies, anti-TFPI antibodies have the potential to be given subcutaneously and at weekly intervals. Three phase 2 trials in HA and HB patients with (HAWI and HBWI) and without inhibitors (HA) have been completed. In the Explorer 4 and Explorer 5 trials,<sup>72</sup> the estimated ABRs in HAWI and HBWI were lower versus HA: 3.0 and 5.9 versus 7.0, respectively. Most inhibitor patients (88.2%) did not escalate the starting dose of 0.15 mg/kg, with potential dose escalation to 0.20 and 0.25 mg/kg in the case of three spontaneous bleeding episodes or more within 12 weeks of treatment. Recently the trials investigating the safety and efficacy of concizumab in hemophilia patients have been paused. The decision was based on the occurrence of thrombotic events, all not fatal, in three patients. One phase 2 trial (BAY1093884) has been also terminated because of thrombotic events.

### Gene Therapy

HA and HB are monogenic, X-linked, coagulation disorders. Gene therapy may provide a long-term correction of the bleeding tendency transferring a functional copy of the gene that is required to express the missing/dysfunctional clotting factor. Several characteristics of hemophilia make the disease a good target for gene therapy: a wide range of factor level is acceptable; relatively low amount of functional protein can provide protection from spontaneous bleeding and avoid the prophylactic administration of factor concentrates; a tight control of factor level is not necessary.

Over the years the efficiency of delivery has markedly improved. The use of adeno-associated viral (AAV) vectors has so far achieved the best results in both preclinical and clinical studies. AAV vectors have been derived from wild-type AAV, a member of the parvovirus family. The specific

characteristics that make AAV vectors a potential first choice for gene therapy is the nonpathogenic nature of wild-type AAV, their weak immunogenicity, and the inability to replicate autonomously. Also, they do not integrate into the host genome. The engineered vectors contain only a residual of the wild-type genetic material and most of it has been replaced with the therapeutic gene cassette. The ClinicalTrials.gov database currently lists a total of 24 active clinical trials evaluating different AAV vectors and lentivirus in HA and HB.

BioMarin funded the first clinical trial for HA, where nine participants with severe hemophilia were enrolled into three dose cohorts using a codon-optimized AAV5 vector containing a B-domain-deleted FVIII gene.<sup>73</sup> BioMarin has also recently submitted a Biologics License Application (BLA) to the U.S. Food and Drug Administration (FDA) for valoctocogene roxaparvovec, its investigational AAV gene therapy, that aims at treating adult patients with HA. AAV vectors have been used in clinical trials for both HA and HB. In HB the infusion of a single dose of AAV8 vector provided long-term therapeutic FIX expression and clinical improvement. This approach, targeting hepatocytes in adult patients with HB, has provided stable expression of FIX protein for more than 6 years.<sup>74</sup> The efficacy of AAV gene transfer has been enhanced through several mechanisms: better design of liver-specific promoters, codon optimization of both F8 and F9 cDNAs, and the use of engineered F8 (B-domain deleted) and F9 (gain-of-function FIX variant R338L) genes.<sup>75,76</sup>

More recently, UniQure has published data on their interim analysis of a 5-year phase 1/2 clinical trial enrolling adults with HB, showing that a single infusion of an AAV5 vector incorporating a gene cassette containing codon-optimized wild-type hFIX resulted in a stable expression of endogenous FIX for up to 1 year of follow-up. Improvement of disease severity could be observed in all participants and allowed eight of nine participants to discontinue FIX prophylaxis.<sup>77</sup> Of note, in contrast to data reported by other trials, no patients presented any immune response to the capsid.

### Challenges to Be Addressed with Novel Therapeutic Approaches to Hemophilia Treatment

Hemophilia patients and treaters have seen the development of several exciting breakthroughs in hemophilia therapies. However, there is a significant gap of knowledge that needs to be addressed in the coming years. There is now evidence from gene therapy clinical trials to consider AAV vector gene transfer an effective option, at least in the short-to-medium term. Long-term efficacy and safety have not yet been assessed and will need to be thoroughly evaluated. The potential loss of factor expression is an important consideration. The impact of liver growth and dilution of transduced hepatocytes in younger patients is not fully understood yet. Possibly, integrating lentivirus-based strategies could overcome these obstacles. Also, it is still unclear whether the immune tolerance induction that has been achieved via AAV transfection in animal models will also translate in humans. This might be important when considering the treatment in patients with high-risk mutations for inhibitors, personal

history of inhibitor, or when patients with active inhibitors are considered for gene therapy. Another restriction could be the presence of already established immunity to the viral capsid, limiting the access to this treatment or a re-dosing. However, at least for some of the gene therapy treatment options (AMT-060, AMT-061; UniQure) it seems that pre-existing neutralizing antibodies to AAV5 did not compromise the sustained production of FIX in three participants.

Novel nonfactor replacement therapies, namely bispecific antibodies with FVIII mimetic properties, and drugs which affect endogenous anticoagulants such as AT and TFPI are attractive alternative approaches to the achievement of hemostasis amongst PwH. In some circumstances though, its use has been associated with thrombosis. Three thrombotic microangiopathy and two venous thromboembolism cases have been reported under prophylaxis with emicizumab. All the thromboembolic events occurred during adjunctive treatment with aPCC for breakthrough bleeds, and specifically the thrombotic risk seems to be related to the high doses used in those cases. One fatal thrombotic event was also reported in a HA patient on a clinical trial with fitusiran, as mentioned above. The management of breakthrough bleedings at the time of concomitant nonfactor replacement therapy will become a more frequent aspect of the activities of hemophilia treaters. Also, there is very little experience with the use of nonfactor product prophylaxis and the perioperative hemostatic management. In these settings, the presence of antidotes could be helpful to effectively and safely restore the blood coagulation. At this time, no antidotes are available, except for recombinant AT, which should be able to reverse fitusiran.

Moreover, data are lacking concerning the effect of new prophylaxis regimens with nonfactor agents on the occurrence of inhibitors, considering variations in terms of timing and intensity of FVIII exposure. The achievement of immunotolerance will remain a goal of treatment, and currently there are ongoing studies aimed at exploring the possibility to achieve FVIII tolerance while obtaining protection from bleeding with emicizumab. If this approach will be proven successful, the patients that have obtained FVIII-specific tolerance could potentially switch back to factor prophylaxis. On the other hand, if the patient is to be maintained on emicizumab alone after the ITI, the duration of the immune tolerance in the absence of regular FVIII infusion is not known and potentially a break in tolerance might occur.

### The Evolution of Laboratory Diagnostics in Hemophilia

With the changing landscape of hemophilia care, the requirements from a laboratory diagnostic perspective have increased substantially. Here, we review the need for specialized coagulation assays in a time when many new hemostatic therapeutics are currently available and will become increasingly available. Of paramount importance is for laboratories to have a communication mechanism in place with clinicians to identify which hemostatic product is being used to facilitate an appropriate testing strategy to best inform clinical decision making.

### Factor Assays and Extended Half-Life Products

Critical and implicit to the proper management of patients with HA and HB is the ability to monitor FVIII and FIX levels, respectively,<sup>78</sup> when patients receive plasma-derived or recombinant factor concentrate. There are two different functional assays to measure factor activity, the one-stage clot-based assay and the chromogenic assay,<sup>79</sup> with the former historically being more widely used.

The clot-based assay measures how patient plasma shortens the activated partial thromboplastin time (aPTT) of FVIII or FIX-deficient plasma. The factor-deficient plasma and the patient sample are preincubated with a contact activator and phospholipids while calcium chloride is later added to initiate fibrin clot formation.<sup>80</sup> The FVIII or FIX concentration in the patient plasma is thus presumably the rate-limiting determining factor of the aPTT.

The chromogenic assay consists of two time-dependent tests that ultimately measure FXa production, which is assumed to be proportional to the amount of FVIII or FIX present in the patient sample that is derived from a standard curve.<sup>79,81,82</sup> Bethesda inhibitor assays, Nijmegen modified or not, are then based on the type of factor assay used in a given laboratory.

With the advent of EHL products the requirements from a diagnostic perspective have changed. Proper monitoring of FVIII and FIX with the EHL products requires chromogenic testing capacity since molecular modification and fusion with the Fc region of immunoglobulin G or with albumin or linkage to polyethylene glycol can lead to either over- or underestimation by approximately 20 to 50% of clotting factor activity level with many one-stage clotting assays due to interaction with the contact activator that varies from laboratory to laboratory.<sup>83,84</sup> However, the chromogenic assays appear to consistently correlate well on external quality assessment of spiked samples with a variety of EHLs.<sup>80</sup> The discrepancy that occurs with aPTT-based assays can be of clinical importance in patients being treated for a major bleed, trauma, or surgery, and thus it is now important to have chromogenic testing capacity to best guide clinical care.<sup>80</sup>

### Laboratory Monitoring of Emicizumab

Dosing of emicizumab is based on weight and can occur at frequency ranging from weekly to monthly. Emicizumab drug concentration assays may prove useful in defining which patients can be converted to less frequent dosing strategies particularly since pharmacokinetic data demonstrate a linear relationship between steady state trough plasma emicizumab concentrations and bleeding rates.<sup>85</sup>

Emicizumab interacts with all intrinsic pathway clotting-based laboratory assays, including all aPTT-based assays, rendering them unreliable and potentially falsely reassuring to the unaware provider. The binding characteristics of emicizumab are such that even low plasma concentrations normalize the aPTT. Under normal circumstances, aPTT-based clotting assays measure the total clotting time of the intrinsic pathway of coagulation, including activation of FVIII to FVIIIa by thrombin; emicizumab does not require activation by thrombin and will therefore result in an exceedingly

short clotting time. Therefore, a one-stage assay performed in the presence of emicizumab would grossly overestimate FVIII:C.<sup>86</sup>

On the other hand, the FVIII chromogenic assay will provide an accurate measure of FVIII activity in a sample containing emicizumab. FVIII chromogenic assay measures the FVIII-dependent activation of FX using purified bovine or human coagulation factor. In the first stage of this assay, patient plasma is added to a reaction mixture that usually contains FIXa, FX, calcium ions, phospholipids, and trace amounts of thrombin. Thrombin triggers the activation of FVIII and the subsequent FIXa-mediated activation of FX. In contrast to the FVIII aPTT-based clotting assay, the addition of thrombin in the first step means that activation of FVIII to FVIIIa is not a major influencing determinant of the assay. FXa production is presumed to be proportional to the FVIII concentration in the plasma sample. Since emicizumab selectively binds to human FIXa and FX, a FVIII chromogenic assay based on bovine factors is recommended.<sup>87</sup>

Neutralizing anti-emicizumab antibody interfering with the drug's hemostatic efficacy has been rarely described,<sup>88</sup> thus the ability to measure emicizumab antibodies using enzyme-linked-immunosorbent serologic assay (ELISA)-based methodology is important in cases where clinical hemostasis is called to question.<sup>87</sup>

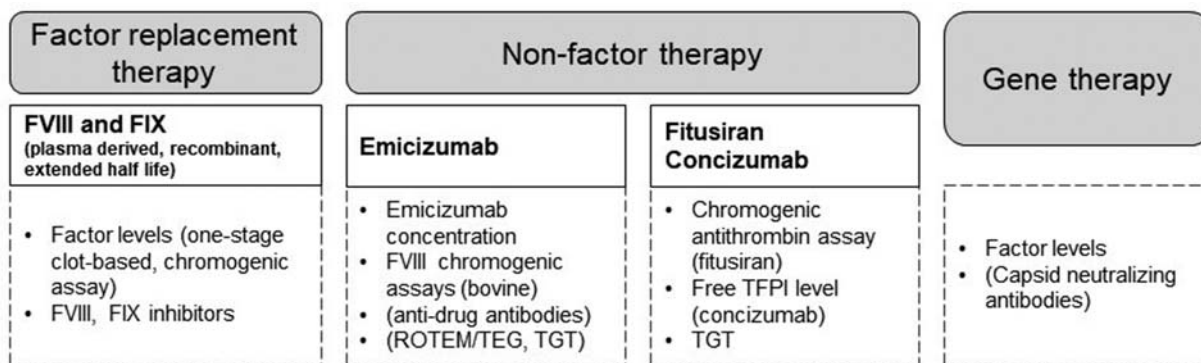
Important consideration must be given to the FVIII assay and Bethesda assay based on it in patients receiving emicizumab, as one-stage aPTT assays will substantially overestimate the FVIII due to the effect of emicizumab on clot-based assays.<sup>85,89</sup> This is an important consideration as patients on emicizumab may occasionally require FVIII replacement for major bleeds or surgeries. Also, others with allo-inhibitors may concomitantly be treated with immune tolerance induction therapy where accurate monitoring of FVIII and FVIII inhibitor levels will be necessary. In these cases, a chromogenic FVIII assay using bovine components must be used as the bovine proteins (FIX and FX) are insensitive to emicizumab while still sensitive to human FVIII rendering ability to measure endogenous or infused FVIII.<sup>90</sup>

### Laboratory Monitoring of Fitusiran and Concizumab

Plasma AT measurements in ongoing clinical trials with fitusiran are measured by a chromogenic AT assay while global hemostasis is being evaluated with an automated thrombogram.<sup>68,69</sup> Concizumab appears procoagulant in vitro as measured by D-dimer and prothrombin fragments as well as global hemostatic assays.<sup>91</sup> The optimal method for monitoring these classes of hemostatic drugs however remains to be determined and should become clearer with ongoing clinical trials.<sup>80</sup>

### Dynamic Assays of Coagulation

Dynamic assays such as thrombin generation and viscoelastic (thromboelastography and rotational thromboelastometry) assays will likely play an increasing role in the care of PwH given the increasing array of products available with hemostatic effect (→ Fig. 1). In fact, the International Society



**Fig. 1** Monitoring of novel hemophilia therapies. FVIII, factor VIII; FIX, factor IX; ROTEM, rotational thromboelastometry; TGT, thrombin generation tests; TEG, thromboelastography; TFPI, tissue factor pathway inhibitor.

on Thrombosis and Haemostasis released a statement supporting the use of viscoelastic assays in clinical management and clinical trials of PwH.<sup>92</sup>

### Gaps of Knowledge

In the era of rapidly evolving therapies for hemophilia, there are new challenges and opportunities for the special coagulation laboratory and clinician. Specialized testing capacity (whether local or remote via reference) is important but only if the test is properly matched to the hemostatic therapeutic and clinical scenario. Thus, effective communication with the laboratory is important now more than ever. The current unknowns or unmet needs from a laboratory perspective include developing a firm understanding of the clinical significance of emicizumab drug levels in the real world, expanding and understanding the utility of dynamic assays of coagulation with novel hemostatic agents as well as solidifying knowledge-translation and exchange efforts to facilitate proper appropriation of laboratory tests for given hemostatic agents.

### Time Capsule

- FVIII/FIX concentrates will remain the standard of care in most patients worldwide and achievement of immunotolerance will still be a goal of treatment.
- Hemophilia-related fatality will decrease in the first years of life thanks to early primary prophylaxis with non-intravenous products.
- Effective communication with the laboratory to ensure proper test appropriation will be critical given the availability of extended half-life factor and nonfactor products.
- Gene therapy will be optimized to achieve long-term factor production and strategies to allow for re-dosing will be devised.

### Conflict of Interest

Dr. Matino reports personal fees from Sanofi, grants and personal fees from Sobi, personal fees from UBS, grants and personal fees from Pfizer, personal fees from Novo-Nordisk, personal fees from BIOVIII, outside the submitted work.

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