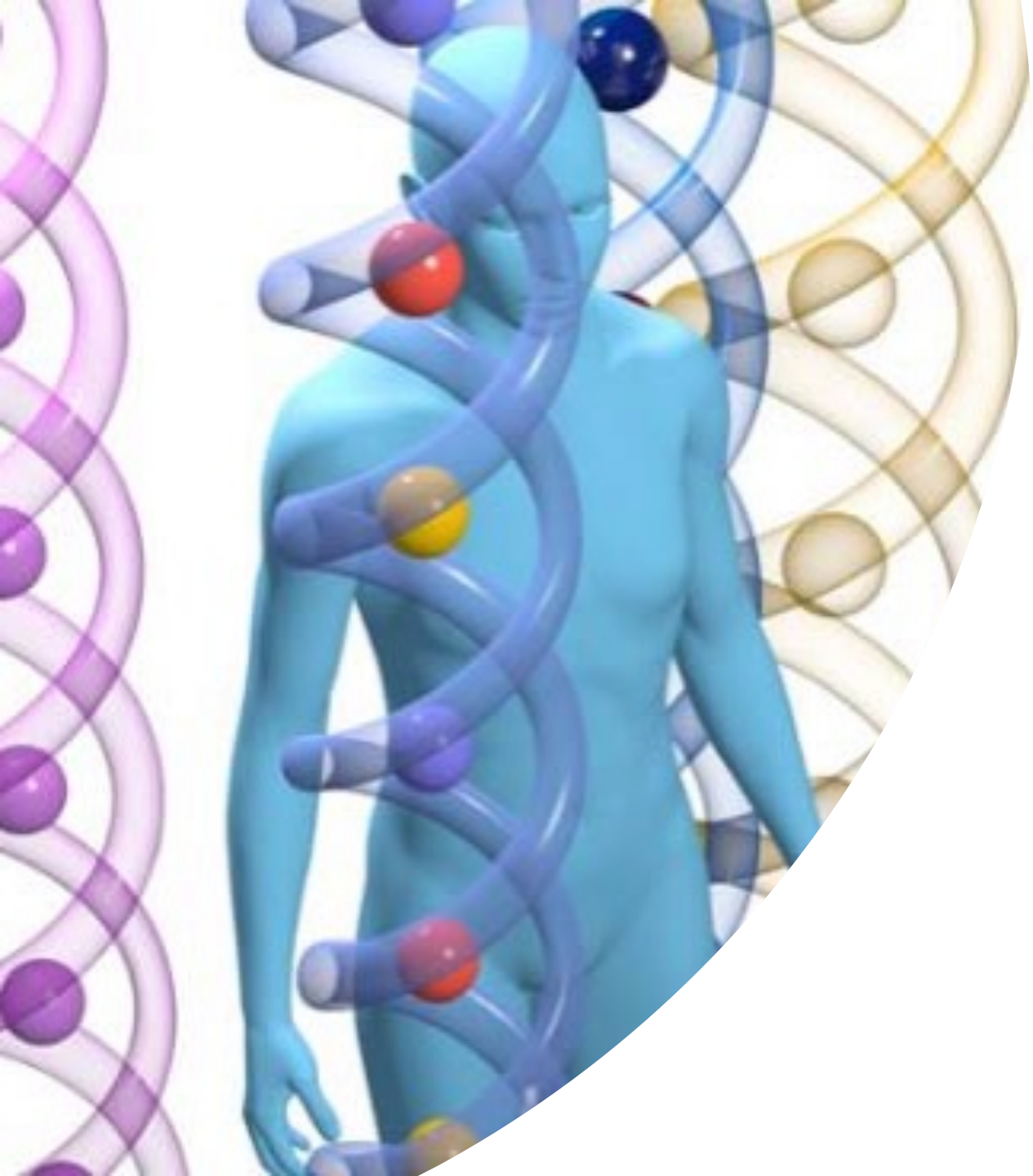

Integrating liver-directed gene therapy into the treatment armamentarium for people with hemophilia.

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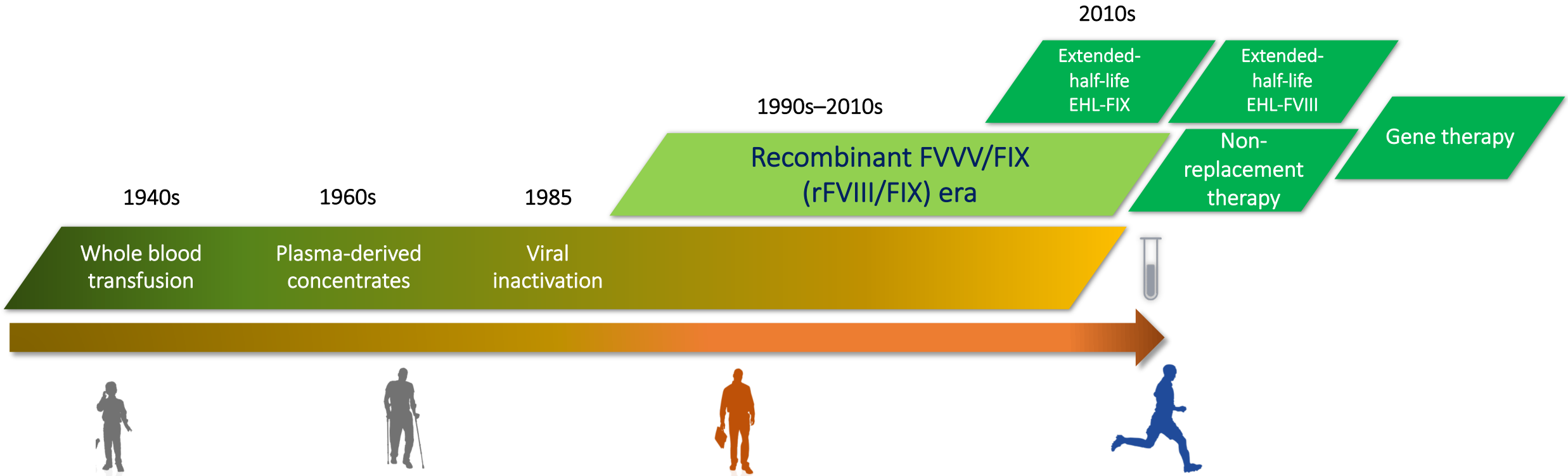
Presenter Disclosures

Speaker or a member of a speaker bureau for:	**Consultant or ad hoc speaker/consultant for:**
BioMarin	BioMarin
Pfizer	Pfizer
CSL Behring	CSL Behring
Takeda	Takeda

The continuing evolution of factor replacement has revolutionized the management of patients with hemophilia (PWH)



1930s
Historically, PWH would not reach adulthood

1960s
Introduction of plasma-derived products dramatically reduced mortality but did not prevent crippling joint damage

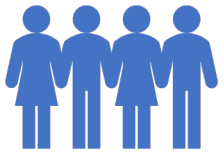
1990s–2010s
Introduction of rFIX-rFVIII improved treatment safety and life expectancy

Today
PWH who have access to treatment can look forward to a normal life expectancy….

Despite Inhibitor formation complications…

Life expectancy for patients with haemophilia: An example of efficacy of prophylaxis and product safety

- Life expectancy for patients with haemophilia has improved as management strategies have improved
  - Italy
    - Life expectancy 71.2 years for 2000–2007 vs 64.0 years for 1990–1999[1]
  - USA
    - Median age of death for non-HIV-infected patients with haemophilia A 72 years between 1995 and 1998[2]
  - The Netherlands
    - Life expectancy 72 years between 1992 and 2001[3]*



***After exclusion of virus-related deaths**

HIV = human immunodeficiency virus

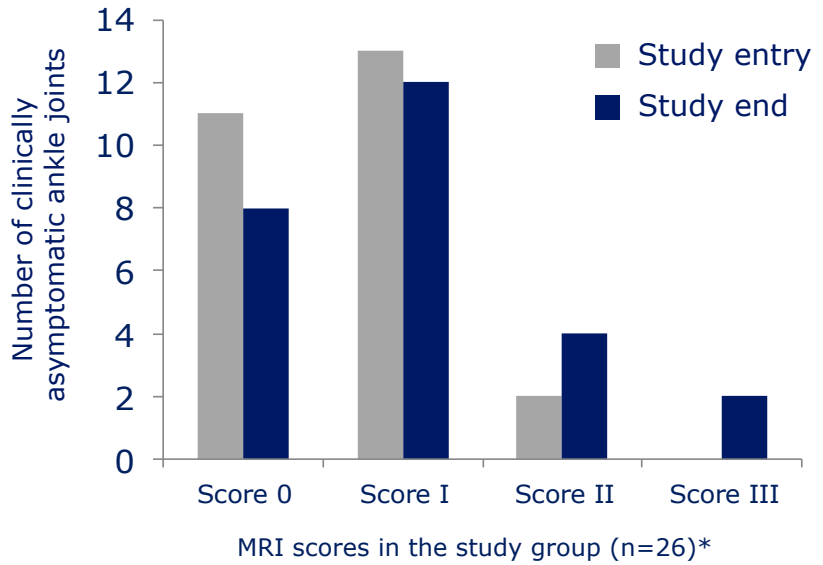
1. Tagliaferri A, et al. *Haemophilia* 2010;16:437–46; 2. Chorba TL, et al. *Am J Hematol* 2001;66:229–40; 3. Plug I, et al. *J Thromb Haemost* 2006;4:510–6.

However,…

Early joint changes in clinically asymptomatic joints despite prophylaxis in hemophilia A and B: a 2000-2010 follow-up

- **Retrospective single Centre study**
  - **26 children on prophylactic treatment from median age of 4**
  - **88% received prophylaxis 2–3 x/week (3 patients on-demand)**
  - **Mean age of the study population at the end of the study was 20 (median 19 years)**
- **Mean observation period: 8 years (median 9 years)**
- **In 18% of patients joint changes detected by MRI despite no bleeds reported**

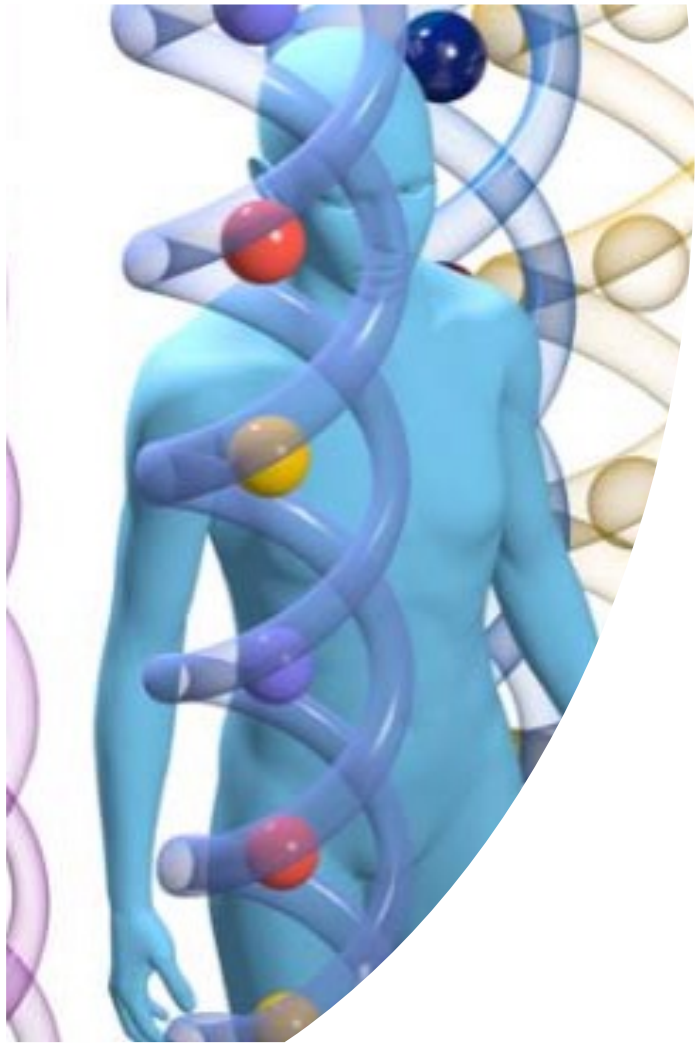
MRI joint scores indicated joint disease in clinically asymptomatic ankle joints despite prophylactic coverage



*MRI scores of clinically asymptomatic ankle joints at study entry and study end; higher score indicates higher abnormality

Haemophilia 2012; 18:369–74.

Replacing/repairing a defective gene: Therapeutic gene transfer (gene therapy)



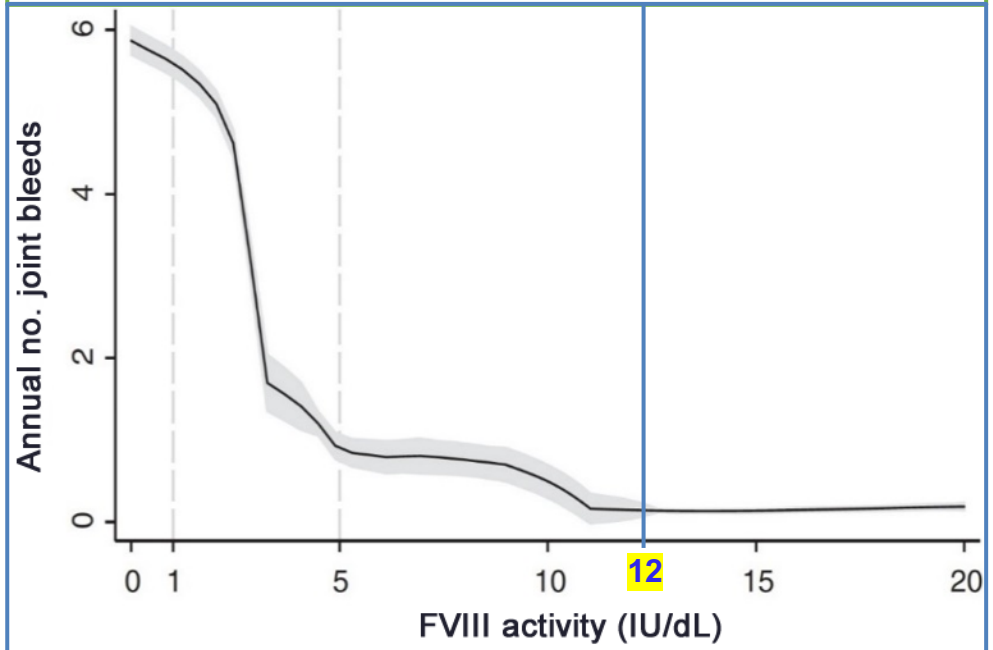
- **Gene therapy:**[1-2]
  - A strategy used to replace or repair a dysfunctional gene
  - Most applicable to diseases caused by a single gene mutation
  - An approach to improve symptoms or potentially cure a disease
  - Administered ex vivo or in vivo, depending on the vector
- The vector (vehicle based on the capsid of a nonreplicating virus) delivers the desired gene to particular “target”.[3]

1.Kumar SR, et al. *Mol Ther Methods Clin Dev.* 2016;3:16034. **2.** Thomas CE, et al. *Nat Rev Genet.* 2003;4(5):346-358. **3.** Mingozzi F, et al. *Blood*. 2013;122(1):23-36.

Objectives of Gene Therapy for Hemophilia[1]

- **Sustained, long-term factor expression** that produces optimal therapeutic levels without troughs
- **Lowest possible dose** that decreases the risk of immune response and lowers manufacturing hurdles/cost
- **Lowest possible frequency of immune response** that minimizes the use of steroids and leads to optimal outcome

Annual number of joint bleeds decreased to near zero at factor VIII activity levels ≥12 IU/dL[2]



Note: Black lines are medians; shaded areas are interquartile ranges.

FVIII=factor VIII.

1. High KA. *J Thromb Haemost*. 2011;9(7):2-11. **2.** den Uijl IE, et al. *Hemophilia*. 2011;17(6):849-853.

In Vivo vs Ex Vivo Gene Therapy depending on the vector. Types of Vectors

3 main types of viral vectors:

- Retroviruses
- Lentiviruses
- adeno-associated viruses (AAVs)

	Retrovirus	Lentivirus	AAV
Genetic material	ssRNA	ssRNA	ssDNA
Tropism	Dividing cells only	Dividing *and* nondividing cells	Dividing *and* nondividing cells
Vector genome forms*	Integrated	Integrated	Non-integrated (Primarily episomal)
Carrying capacity	8 kb	8 kb	<5 kb
Use in gene therapy	Hemoglobinopathies, ADA	Hemoglobinopathies, ADA	Hemophilia, SMA (SMN-1), DMD, LCA

* **Abbreviations:** AAVs=adeno-associated viruses; ssRNA=single-stranded RNA; ssDNA=single-stranded DNA; kb=kilobase, SMA (spinal muscular atrophy); DMD (Duchenne muscular dystrophy) ; LCA (Leber congenital amaurosis). AAV-directed gene therapy is also used for retinitis pigmentosa, hemophilia, and lysosomal storage disorders (LSDs).

Duration of expression and long-term efficacy of Factor IX following gene delivery to hepatocytes by AAV vectors.

Factor IX									
AAV vector/gene/capsid **ref**	**Men investigated (n)** dose administered	**Observation period (Yrs, median)**	**Peak factor activity over time (%, mean, median), expression durability** After 1 year	After 2 years	After 3 years	After 4 years	After 5 years	**Annualized bleeding rate (ABR) before vs after vector administration)** ABR reduction (% Events per year, median)	**Comments**
AAV8-FIX /FIX/ AAV8 **N Engl. J Med. 2014; 371(21): 1994–2004.** **Blood (2018) 132, Suppl. 1, 491.**	10 2×10^{11} vg/kg 6×10^{11} vg/kg 2×10^{12} vg/kg	10 *	3 different doses, stable transgene expression 2×10^{11} vg/kg (n=2) → 1.8±0.7% 6×10^{11} vg/kg (n=2) → 2.5±0.9% 2×10^{12} vg/kg (n=6) → 5.1±1.7%					90 (94% in the 2×10^{12} vg/kg group) (from: 15.5 [range 10.3 to 19.3] to 1.5 [Range 1.0 to 4.0]) after vector administration	annual FIX use of FIX concentrate dropped by 2/3, annual bleeding rate declined by 82%
AMT-060/FIX/AAV5 **Blood 2018 1;131: 1022-1031**	5 5×10^{12} vg/kg	4	4.4 IU/dL Stable transgene expression					54% reduction of ABR (From: 9.8% to 4.6%)	8 of 9 participants receiving FIX at study entry stopped prophylaxis
	5 2×10^{13} vg/kg	4	6.9 IU/dL, Stable transgene expression					50% decrease (From: 4.0 to 2.0)	
SPK-9001/FIX-R338L/ SPK100 **N Engl. J Med. 2017 Dec 7; 377(23): 2215–2227.**	10 5×10^{11}vg/kg	3-4	33.7±18.5% (Range, 14-81) Stable transgene expression					94.5% (From: 11.1 [range, 0- 48] to 0.4 [range, 0-4] after vector administration	8/10 participants (80%) had zero bleeds.
rAAV-Spark100-hFIX-Padua **Blood (2021) 138 (Suppl. 1): 3975.** §§	13	5	22.8§	25.4§	22.9§	24.9§	19.8§	ABR 0-0.9 over the course of follow-up. No patient resumed FIX prophylaxis. Four patients underwent 6 surgical procedures during the follow-up. The 2 emergent procedures were performed without the need of additional FIX.	No patient developed an inhibitor or had a thrombotic event. Liver ultrasounds revealed steatosis only in one patient.

Factor IX Padua (FIX-R338L mutation) was employed in more recent studies on HB.

* Only minimal evidence of a decline in plasma FIX levels have been shown after a single IV Factor IX gene transfer.

§ One stage Factor assay.

§§ Overall, this is the largest cohort of HB patients with a follow-up duration up to 5 years following treatment with an adeno-associated virus gene therapy expressing a highly active variant of FIX.

Di Minno G. et al, *Blood Rev. 2022 Aug 23:101011.*

Duration of expression and long-term efficacy of Factor VIII following gene delivery to hepatocytes by AAV vectors.

Factor VIII									
AAV vector/gene/capsid° ref	**Men investigated (n)**	**Observation period (Years, median)**	**Peak factor activity over time (%, mean, median), expression durability**					**Annualized bleeding rate (ABR) before vs after vector administration)**	**Comments**
	dose administered		**After 1 year**	**After 2 years**	**After 3 years**	**After 4 years °°**	**After 5 years**	**ABR reduction (% Events per year, median)**	
BMN- 270-201 phase 1-2 study # BDD-FVIII/AAV5-(hFVIII-SQ) N Engl. J. Med. 2020; 382:29-40. Haemophilia. 2021; 27:947–956.	7 6×10^{13} vg/kg	5	64.3 (60.3) § (Basal: 4%)	36.4 (26.2) §	32.7 (19.9) §	24.2 (16.4) §	11.6 (8.2) §	96 (16.3±15.7 before vs 0.7±1.6 after vector administration; median, 16.5 vs 0 events/yr)	6/7 participants (86%) had zero bleeds. Declining transgene expression°°
	6 4×10^{13} vg/kg	3	21.1 (23.8) (<3-40) § (Basal: 6%)	12.3 (11.6) (<3-20) §	10.2 (7.3)	-	-	92 (12.2±15.4 before vs 1.2±2.4 after vector administration; (median, 8 vs 0 events/yr)	4/6 participants (67%) had zero bleeds. Declining transgene expression
GENEr8-1 phase 3 study BDD-FVIII/AAV5-(hFVIII-SQ) New Engl. J Med 2022, 386;11, 1013-1025	134 6×10^{13} vg/kg	5	42.8 (23.9)	23 (11.8)	16.8 (9.3) (In 17 PWH)	-	-	85% (4.8 [2.8] vs 0.9, 0.7, and 0.6 during years 1 and 3, after vector administration)	98% reduction of FVIII infusions vs baseline (p-value <0.0001); 84% zero treated bleeds, 95% off prophylaxis
SPK-8011/ BDD-FVIII/LK03 N Engl. J Med. 2021; 385: 1961–1973.	16 5×10^{11} vg/ kg 1×10^{12} vg/kg 2×10^{12} vg/kg	2-4 (Range, 5.5-50.3 months) in 12 individuals	12.9±6.9% vs. 12.0±7.1%§§ (mean ±SD) stable transgene expression of the 3 doses* 5×10^{11} vg/kg 8-12%; follow-up: 2,5-3.3 years 1×10^{12} vg/kg 3-22%; follow-up: 2-2.5 years 2×10^{12} vg/kg 0-25%; follow-up: 1-1.5 years					91.5 8.5 (range, 0-43.0) before vs 0.3 (range, 0-6.5) after vector administration	60-100% of participants with 0 bleeds (years 1 through 4, mean: 39%). Prophylaxis always discontinued.

#An AE of a salivary gland carcinoma not attributed to treatment was reported in a participant of Phase1/2 trial[158].

° B -domain-deleted FVIII (BDD-FVIII) was the gene employed in all the studies on liver-directed gene therapy for HA.

°° a median factor VIII expression of 16 IU/dl likely approaching a sustained plateau was found at the 4-yr follow-up for the 6×10^{13} vg/kg dose (the anticipated licensed dose).

* No clear dose response in steady-state factor VIII activity was observed with the 3 doses, nor any apparent decrease in factor VIII activity over time.

§ chromogenic assay.

§§one stage Factor assay. One-stage factor VIII assay provides data ≈1.6 times as high as those determined with the use of a chromogenic factor VIII assay. For instance, by one-stage assay, the mean (median) FVIII activity level at the end of year 5 in the BMN- 270-201 study was 18.7 (15.7) IU/dL.

Duration of expression and long-term efficacy of Factor VIII following gene delivery to hepatocytes by AAV vectors.

AAV vector/gene/capsid° ref	Men investigated (n) dose administered	Observation period (Years, median)	Factor VIII: Peak factor activity over time (%, mean, median), expression durability — After 1 year	After 2 years	After 3 years	After 4 years °°	After 5 years	After 6 years	Annualized bleeding rate (ABR) before vs after vector administration) ABR reduction (% Events per year, median)	Comments
BMN- 270-201 phase 1-2 study # IU/dL BDD-FVIII/AAV5-(hFVIII-SQ) *N Engl. J. Med. 2020; 382:29-40.* *Haemophilia*. 2021; 27:947–956.	7 6×10^{13} vg/kg	5-6	64.3 [60.3] § (Basal: 4%)	36.4 [26.2] §	32.7 [19.9] §	24.2 [16.4] §	11.6 [8.2] §	9.8 [5.6] IU/dL§ °°°	96 *(16.3±15.7 before vs 0.7±1.6 after vector administration; median, 16.5 vs 0 events/yr)*	6/7 participants (**86%**) had zero bleeds. Declining transgene expression°°
	6 4×10^{13} vg/kg	3	21.1 (23.8) (<3-40) § (Basal: 6%)	12.3 (11.6) (<3-20) §	10.2 (7.3)	-	-	-	92 *(12.2±15.4 before vs 1.2±2.4 after vector administration; (median, 8 vs 0 events/yr)*	4/6 participants (**67%**) had zero bleeds. Declining transgene expression
GENEr8-1 phase 3 study BDD-FVIII/AAV5-(hFVIII-SQ) *New Engl. J Med 2022, 386;11, 1013-1025*	134 6×10^{13} vg/kg	5	42.8 (23.9)	23 (11.8)	16.8 (9.3) (In 17 PWH)	-	-	-	85 *(4.8 [2.8] vs 0.9, 0.7, and 0.6 during years 1 and 3, after vector administration)*	98% reduction of FVIII infusions vs baseline (p-value <0.0001); 84% zero treated bleeds, 95% off prophylaxis
SPK-8011/ BDD-FVIII/LK03 *N Engl. J Med. 2021; 385: 1961–1973.*	16 5×10^{11} vg/ kg 1×10^{12} vg/kg 2×10^{12} vg/kg	2-4 (Range, 5.5-50.3 months) in 12 individuals	12.9±6.9% vs. 12.0±7.1%§§ (mean ±SD) stable transgene expression of the 3 doses* 5×10^{11} vg/kg **8-12%;** follow-up: 2,5-3.3 years 1×10^{12} vg/kg **3-22%**; follow-up: 2-2.5 years 2×10^{12} vg/kg **0-25**%; follow-up: 1-1.5 years						91.5 *8.5 (range, 0-43.0) before vs 0.3 (range,* *0-6.5) after vector administration*	**60-100%** of participants with 0 bleeds (years 1 through 4, mean: 39%). Prophylaxis always discontinued.

Di Minno G. et al, ***Blood Rev. 2022 Aug 23:101011.***

Long-Term safety data (12-15 years) in the first cohort of patients who received systemic intravenous AAV for delivery of FIX gene to hepatocytes (AAV2-hFIX16, 8 x10^{10}-2x10^{12} vg/kg). §

Pre-vector infusion			Follow-Up		
Subjects evaluated (Vector Dose vg/kg)	**Age (years) at Vector Infusion**	**Co-morbidities Prior to Vector Infusion**	**Age (years) at follow-up**	**Follow-up Duration (years)**	**Co-morbidities at follow-up**
Subject B (8 x10^{10})	48	HCV, liver fibrosis (F1,) ** HIV, hemophilic arthropathy* non-Hodgkin's lymphoma***	62	14	Atrial fibrillation, hypertension, end-stage kidney disease, NIDDM, cataracts, HIV, hemophilic arthropathy, progressive*
Subject D (4x10^{11})	20	HCV, F1 liver fibrosis** HIV, Gilbert's disease, headaches, migraine, hemophilic arthropathy*	33	13	HCV, Gilbert's disease, headaches, migraine with dizziness, HIV, hemophilic arthropathy, progressive, * carpal tunnel, bilateral* lipodystrophy
Subject G (4 x 10^{11})	27	Hemophilic arthropathy*	40	12.5	Hemophilic arthropathy, progressive*
Subject E (2 x10^{12})	31	Homophylic arthropathy*	46	15	Hemophilic arthropathy, progressive* fatty liver infiltration

Abbreviations: vg, vector genomes; HCV, hepatitis C virus; HIV, human immunodeficiency virus; F1, fibrosis stage 1; NIDDM, non-insulin-dependent diabetes mellitus.

Notes: § Modified from: George LA et al Mol Ther. 2020 Sep 2;28(9):2073-2082. In all cases, expression from the vector was subtherapeutic or limited in duration due to a cellular immune response to the AAV capsid, which prevented analysis of expression durability and long-term efficacy.

*Co-morbidity deemed related to underlying hemophilia.

**Metavir score determined by liver biopsy. Repeat liver biopsies post-vector were not completed due to ethical concerns of performing a procedure in severe HB subjects at risk of bleeding and no direct benefit,

***Subject B was diagnosed with non-Hodgkin's lymphoma and treated with chemotherapy and radiation prior to vector infusion and remained in remission throughout the duration of follow-up.

Long-Term safety data

Animal studies

Statement	Refs.
HCC in mice after systemic delivery of rAAV gene therapy vector for treatment of mucopolysaccharidosis type VII.	A. Donsante, et al, Science 317(5837) (2007) 477.
-HCC in mice with inborn errors of metabolism several months after neonatal injections of high AAV vector doses -HCC associated with vector integration and overexpression of microRNA-341 proximal to the RNA imprinted and accumulated in nucleus (*Rian*) locus. -No insertional mutagenesis and cancer in species without microRNA-341 (e.g., non-human primates, dogs).	R.J. Chandler, et al, JCI 125(2) (2015) 870-80. R.J. Chandler, et al, Mol. Ther. 24(2) (2016) 198-201.
- >95% of AAV vector copies persist in dog liver as episomal, non-integrated forms. - AAV vector integrations in liver cells ranges ≈1/1,000- 1/10,000 cells. - >90% of integrated vectors in intergenic regions of the genome. - Non-random location of the integrated vectors, some genomic sites being prone to repeated vector insertions.	-P. Batty, D. Lillicrap, Hemasphere 5(3) (2021) e540.
AAV integration is dose-dependent	Dalwadi D.A, et al Mol. Ther. 29(10) (2021) 2898-2909.
AAV integration occurs in active transcription sites	-Nault J.C. et al, Nat Genet 47(10) (2015) 1187-93. -R.J. Chandler, et al, JCI 125(2) (2015) 870-80. -Nakai H. et al, Nat Genet 34(3) (2003) 297-302.
-Clonal expansion of cells with insertions near genes potentially associated with growth control in 2/9 HA dogs >10 years after AAV8 or AAV9-transgene delivery expressing functional canine factor VIII.* -No evidence of malignancy (i.e., overt nodule formation or transformation) or abnormal liver function.*	Nguyen G.N, et al, Nat Biotechnol 39(1) (2021) 47-55

* >10 years after AAV8 or AAV9-transgene delivery expressing functional canine factor VIII

Gene therapy studies for Haemophilia A and Haemophilia B: Stringent exclusion criteria employed

Exclusion Criterion	Expected % of HA and HB patients excluded
Patients under 18 years of age	
Patients with less than 30–50 exposure days to FVIII/FIX concentrates	
Current or prior history of inhibitors to FVIII or FIX	up to 30% of patients with HA and in 5% of those with HB
Underlying liver disease or active viral hepatitis B or C infections	~1/3 of cases in haemophiliacs over the age of 35
Presence of neutralizing antibodies (NAbs) to AAV serotype higher than a cutoff caused by natural AAV infection	Depending on the serotype, ~30-40% of the haemophilia as well as of the general population

Based on these exclusion criteria, presently the vast majority of patients with hemophilia is not eligible for liver-directed gene therapy.

«Attempts to eliminate pre- existing NAbs only slightly decreased titers and did not eliminate the Nabs».

Doshi BS and Arruda VR. *Ther Adv Hematol* 2018, Vol. 9(9) 273–293

Older Approaches	References
Immunosuppression	-Corti M et al. *Mol Ther Methods Clin Dev* 2014; 1 -Unzu C *et al*. *J Transl Med* 2012; 10: 122.34,35
Plasmapheresis	-Monteilhet V *et al*. *Mol Ther* 2011; 19: 2084–2091.36
Inclusion of empty capsids ***(to serve as decoys)***	-Mingozzi F *et al*. *Sci Transl Med* 2013; 5: 194ra192
Localized vector infusion	-Mimuro J *et al*. *Mol Ther* 2013; 21: 318–323

Newer Approaches	References
Immunosuppression ***(imlifidase)***	- Leborgne, C. et al. *Nat. Med* 26(7) (2020) 1096-1101
HB: Little inhibitory effect on transgene expression of pre-existing anti-AAV5 Nabs	- Majowicz A., et al. *Mol. Ther. Meth. Clin. Devel.*14 (2019) 27-36. - Miesbach W. *Haemophilia* 28(S1) (2022) 25-126.

- Support to this finding stems from the lack of correlation between FIX activity and titer of pre-existing AAV5 NAbs (up to a titer of 678) in individual participants from the phase III AMT-061 study
- (NCT03569891). A dose-finding trial for HA patients with FVIII inhibitors are ongoing (NCT03734588, NCT04684940).

Di Minno G. et al, *Blood Rev. 2022 Aug 23:101011.*

Etranacogene dezaparvovec results in similar FIX activity levels, in the presence and absence of AAV5 Nabs. Baseline characteristics

Phase 3

Characteristic	Full analysis set (N=54)
Mean age, years (SD, range)	41.5 (15.8, 19–75)
Severity of hemophilia B at diagnosis, n (%) Severe (FIX <1%) Moderately severe (FIX ≥1% and ≤2%)	 44 (81.5) 10 (18.5)
Positive HIV status, n (%)	3 (5.6)
Prior hepatitis B infection, n (%)	9 (16.7)
Prior hepatitis C infection, n (%)	31 (57.4)
Pre-screening prophylaxis, n (%) EHL-FIX SHL-FIX	 31 (57.4) 23 (42.6)
Detectable AAV5 NAbs at baseline, n (%, max titer)	23 (42.6, 3212.3)
0 bleeds in lead-in, n (%)	16 (29.6)
Cumulative bleeds in lead-in, n	123

AAV, adeno-associated virus; EHL, extended half-life; FIX, clotting factor IX; HIV, human immunodeficiency virus; NAbs, neutralizing antibodies; SD, standard deviation; SHL, standard half-life.

1. Pipe SW, et al. Oral presentation at 62nd ASH meeting, December 2020. 2. Pipe SW, et al. Poster presented at ISTH congress, July 2021.

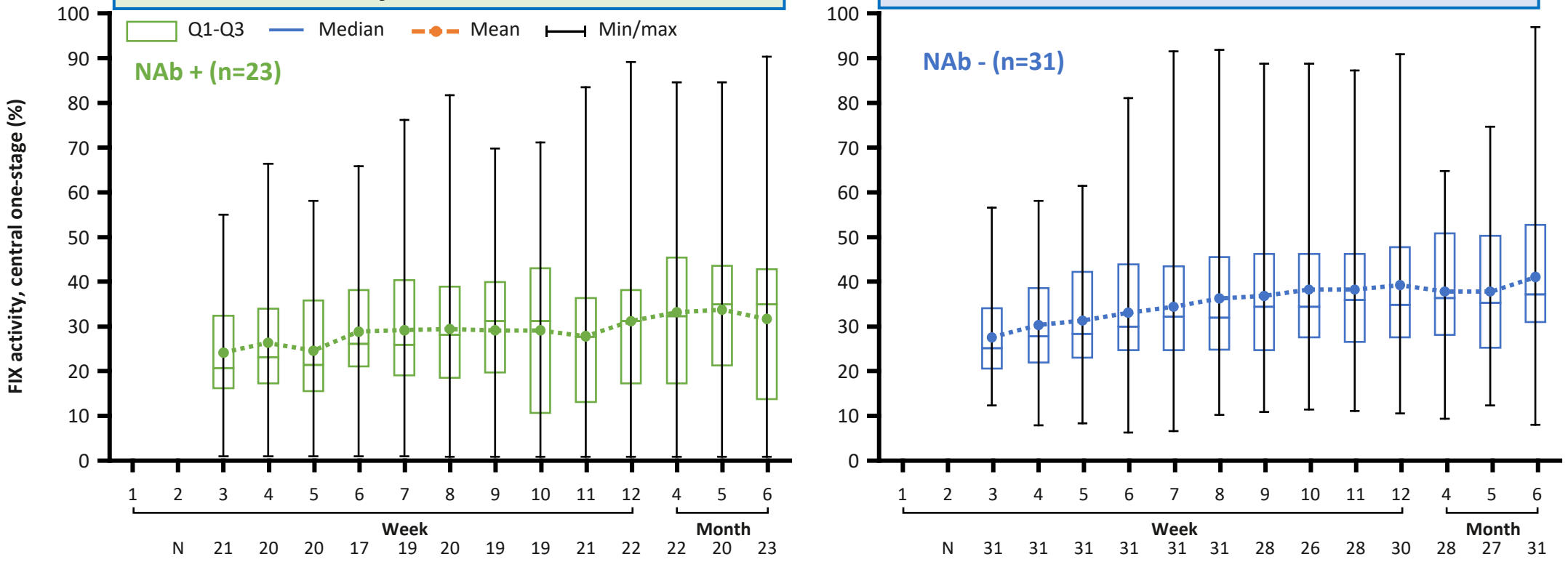
Etranacogene dezaparvovec results in similar FIX activity levels, in the presence and absence of AAV5 Nabs: 6-mo data

Phase 3 6-mth data

Presence of NAbs vs week 26 FIX activity*

For participants **with** pre-existing NAbs, **mean FIX activity was 32.7 IU/dL**[1]

For those **without** pre-existing NAbs, **mean FIX activity was 41.3 IU/dL**[1]



There was no significant difference in baseline characteristics between patients with or without AA5 NAbs.

* Uncontaminated central laboratory data (the visit did not occur within 10 days of exogenous FIX use). FIX levels beginning with the week 3 assessment were used in the analysis.

AAV, adeno-associated virus; FIX, factor IX; IU, international unit; NAb, neutralizing antibody. Leebeek FWG, et al. Oral presentation at ISTH congress, July 2021.

Risk of inhibitor formation to vector-derived clotting factors, I.

- **Immune tolerance induction (ITI) is the standard of care in patients with high-titer inhibitors.[1] However:**
  - ✓ **to avoid life-threatening immune complex-associated nephrotic syndrome and anaphylaxis during ITI, long-term management with rFVIIa infusions is preferred in HB patients.[2]**
  - ✓ **emicizumab is the standard of care to prevent bleeds in patients with inhibitors; in those waiting for ITI or failing it, and maybe during ITI.[3]. However, lack of data on long-term outcomes of emicizumab and the chance to use FVIII (when the titre is very low) for prophylaxis, emergency bleeds and surgery, argue for restoring FVIII use in these patients.[4]**

- **So far, no inhibitor formation has been reported in subjects enrolled in HA or HB gene-therapy studies to date.**
  - ✓ **This is also true for studies that employed the FIX Padua variant (4-yr follow-up in the SPK-9001 HB gene therapy study)**
  - ✓ **However, exclusion and inclusion criteria only allowed enrollment in gene therapy studies of patients with the lowest tendency to develop an inhibitor (PTPs with ED > 150 and no evidence of past inhibitor formation).**

- **Tolerance for clotting factors also emerges in hemophilia dogs following long-term FVIII/FIX expression by rAAV-mediated liver-directed gene therapy.**

- **Rather than inhibitor formation, canine data support tolerance to transgene products after long-term FVIII/FIX expression by rAAV-mediated liver-directed gene therapy. Inhibitor eradication has also been achieved when an immune response to FVIII/FIX was already present [5-10].**

- **A dose-finding trial in HA patients with FVIII inhibitors is ongoing (NCT03734588).**

1. M. Carcao, et al, Haemophilia 25(4) (2019) 676-684; 2. D. DiMichele, British journal of haematology 138(3) (2007) 305-15; 3. R. Ljung, et al, European journal of haematology 102(2) (2019) 111-122, 4.G. Di Minno, et al, Haemophilia 28(1) (2022) 55-64.; 5. F. Mingozzi, et al, Blood 110(7) (2007) 2334-41; 6. A. LoDuca, et al., Current gene therapy 9(2) (2009) 104-14; 7. J.D. Finn ,et al, Blood 116(26) (2010) 5842-8; 8. J.D. Finn, et al, Blood 120(23) (2012) 4521-3; 9. J.M. Crudele, et al, Blood 125(10) (2015) 1553-61; 10 V.R. Arruda, et al, JTH 14(6) (2016) 1121-34.

Risk of inhibitor formation to vector-derived clotting factors, II.

The Italian ITI Registry (137 Patients): characteristics at ITI start

Variable	median	Range (or %)
Age at inhibitor diagnosis, yrs	2.5	0.1-56.9
Exposure days to FVIII, n	15	3 - >500
Age at ITI start , yrs*	4.8	0.3-58.5
< 8 yrs, n	82	59.9%
Interval (inhibitor diagnosis-ITI), mo	19	<1 – 332
< 24 mo, n	78	56.9%
Inhibitor titer at ITI start, BU/ml	4.5	0 – 200
< 10 BU/ml, n	105	77.3%
< 5 BU/ml, n	73	53.2%
Historical peak titer, BU/ml	64	6 – 920
< 200 BU/ml, n	105	76.6%

Di Minno G. , et al, Haemophilia 28(1) (2022) 55-64.

Risk of inhibitor formation to vector-derived clotting factors, III.

•Very young patients are a large proportion of the hemophilia population with inhibitors.[1]

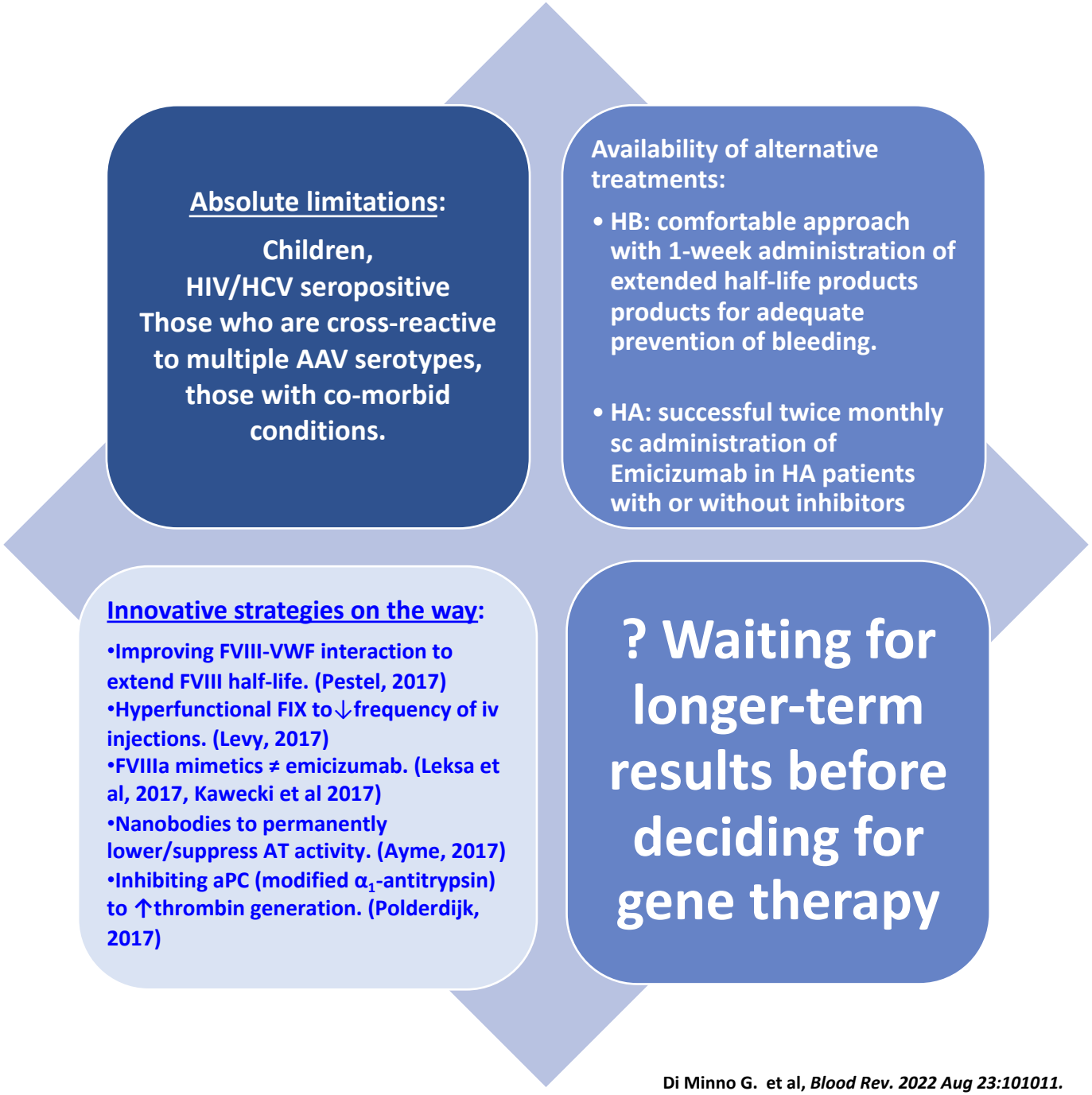
•The immaturity of the immune system and the low likelihood of detectable NAbs titers 2,3], make the first years of age as an ideal time for liver-directed gene therapy in hemophilia.[4] However, the likely loss of efficacy is a major drawback of gene therapy performed at early age.

•The human adult liver is 16 times heavier than the neonatal one.[5] Due to liver growth and because of AAV vector genome dilution in transduced hepatocytes, it is unlikely that a neonatal or early in life injection will suffice to provide sustained correction of the hemophilia.

•Moreover, being presently unclear whether titers of preexisting antibodies against vectors should be considered for newer transgene expressions [6] or whether a second dose with the same or other AAV vectors is conceivable, alternative strategies such as integrating vectors (e.g., lentiviral vectors) [7] or locus-specific genome engineering should be considered.

1. G. Di Minno, et al, Haemophilia 28(1) (2022) 55-64; 2. J. Mimuro, et al, J. Med Virol 86(11) (2014) 1990-7; 3. V. Louis Jeune, J, et al, Hum. gene ther meth 24(2) (2013) 59-67; 4 T.R. McKay, et al, Curr. pharmaceutical des. 17(24) (2011) 2528-41; 5. J.M. Coppoletta, et al, Am. J. Path. 9(1) (1933) 55-70; 6. K.L. Gollomp, et al, Transf. Aph Sci. 58(5) (2019) 602-612; 7. M. Milani, et al, Science translational medicine 11(493) (2019).

Toward the integration of gene therapy into the treatment armamentarium of people with hemophilia.



Di Minno G. et al, *Blood Rev. 2022 Aug 23:101011.*

Outcomes of Clinical Trials with Extended Half-Life (EHL) FVIII/FIX Concentrates.

§ Data from: *J Clin Med 2017 Mar 28;6(4):39, Haematologica 2020, 105(3):545-553, Blood. 2021 Apr 22;137(16):2231-2242, Semin Thromb Hemost. 2021 Feb;47(1):32-42*

EHL factor IX products.					
Engineered protein	**Plasma half-life (hours)**	**Half-life prolongation***	**References**	**Comments**	**EHL factor IX products. Major findings**
Efrenonacog alfa	82	4.3	-N. Engl. J. Med. 2013; 369:2313–2323, -Lancet Haematol. 2017;4: e75–e82, -Thromb Haemost. 2017 Feb 28;117(3):508-518.	-zero bleed in 33% of patients. -median ABR for all bleeds= 2.0 (6-9-fold decrease compared to episodic treatments). -median ABR for spontaneous joint bleeds= 0.	-4-6-fold increase in half-life* -Weekly/ Every 2 weeks prophylaxis -↓60% of numbers of infusions -Through levels 5-10 IU/dl
Albutrepenonacog alfa	101	5.3	Blood. 2016; 127:1761–1769, Thromb. Haemost. 2016; 116:659–668.	-median spontaneous ABR = 0 -100% resolution of target joints	
Nonacog beta pegol	93	4.9	Blood. 2014; 124:3880–3886. J. Thromb. Haemost. 2016; 14:1521–1529.	-67% in the 40 IU/kg arm and 7% in the 10 IU/kg arm had resolution of target joints. -median ABR for all bleeds: 1.0 (15.5-fold decrease compared to on-demand treatment).	
EHL factor VIII products.					
Engineered protein	**Plasma half-life (hours)**	**Half-life prolongation****	**References**	**Comments**	**EHL factor VIII products. Major findings**
Efmoroctocog alfa	19	1.5-1.7	Blood. 2014; 123:317–25, J Thromb Haemost. 2015; 13:967–77, Haemophilia. 2016; 22:72–80.	-zero bleeds in 17.4% adults and 46% kids. -median ABR for all bleeds= 0.66-(kids)-3.6 (adults) (9-10-fold decrease compared to episodic treatment).	-1.3-1.7-fold increase in half-life -Weekly/ twice weekly prophylaxis -Infusion numbers: ↓30% Weekly/ twice-weekly prophylaxis -Through levels: 2-3 IU/dl
Rurioctocog alfa pegol	14.3	1.3-1.5	Blood. 2015; 126:1078–85, Haemophilia 2017;23(02): 238–246, Blood. 2021 Apr 1;137(13):1818-1827, Haemophilia. 2020 Jul;26(4): e168-e178.	-zero bleeds in 39.6% adults and 38% kids. -median ABR for all bleeds= 2.0 (kids), 1.9 (adults) (20-fold decrease compared to episodic treatment).	
Damoctocog alfa pegol	19	1.6	J Thromb Haemost. 2017; 15:411–19., Haemophilia 2020 May;26(3): e55-e65 Haemophilia 2019;25(06):1011–1019	-zero bleeds in adults: 37.2% (weekly prophylaxis) and 45.5% twice weekly prophylaxis. No data in kids. -median ABR for all bleeds in adults: 3.9 (weekly prophylaxis); 1.9 (twice weekly prophylaxis); 2.9 in kids across all treatment arms (20-fold decrease compared to episodic treatment).	
Turoctocog alfa pegol	18.4	1.6	Thromb Haemost 2017; 117:252–61. Thromb Haemost.2017; 117:1705–13, Haemophilia.2019; 25:373–81	-zero bleeds (every 4 days prophylaxis): 40% (adults), 42.6% (kids) -median ABR for all bleeds (every 4 days prophylaxis): 1.2 (adults), 1.94 (kids) (15-fold decrease compared to on-demand treatment).	

***Calculated from an average plasma half-life of standard FIX products of approximately 19 hours. **Calculated from an average plasma half-life of standard coagulation FVIII of approximately 12 hours.**

Bleeding rates observed with different dosing (1.5-6 mg/kg) and scheduling (once a week-once a month delivery) regimens of subcutaneous emicizumab in HA patients with or without inhibitors in the context of the HAVEN studies.

Study protocols		Efficacy (Primary analysis)		Long-term efficacy (Pooled analysis) Blood. 2021 Apr 22;137(16):2231-2242.		Emicizumab: Major findings/ Comments.
STUDY	**PROPHYLACTIC DOSING REGIMENS (NUMBERS OF PATIENTS EXAMINED)**	**Treated bleeds * (MEDIAN)**	**ZERO BLEEDING (RATES)**	**121-144 wks Treated bleeds (MEDIAN)**	**121-144 wks % ZERO BLEEDING (RATES)**	- Subcutaneous route of administration (ease-of-use) -Prophylaxis also effective in previously untreated patients (PUPs) - Weekly prophylaxis also efficacious in patients with inhibitors. - 2-4-week prophylaxis, efficacious in patients without inhibitors (further reduction of treatments as compared to EHL products) - Good safety profile when used in combination with rFVIIa or FVIII -Warning when used in combination with FEIBA in patients with inhibitors to FVIII - Black box warning (FDA) for thrombosis
HAVEN 1 (n=53) § **Adults/adolescents > 12 yrs with FVIII inhibitors**	Once weekly (1.5 mg/kg) (n=35)	2.9	63%	0.7	89.2	
	No prophylaxis (n=18)	23.3	6%			
HAVEN 2 (n=88) §§ **Paediatric < 12 yrs with FVIII inhibitors**	Once weekly (1.5 mg/kg) (n=68)	0.3	76.9%	0.4	82.4	
	Every 2 weeks (3.0 mg/kg) (n=10)	0.2	90%			
	Every 4 weeks (6 mg/kg) (n=10)	2.2	60%			
HAVEN 3(n=89) §§§ **Adults > 12 yrs without FVIII inhibitors**	Once weekly (1.5 mg/kg) (n=36)	1.5	50%	0.7	82.0	
	Every 2 weeks (3.0 mg/kg) (n=35)	1.3	40%			
	No prophylaxis (n=18)	38.2	0			
HAVEN 4 (n=41) §§§§ **Adults > 12 yrs with/without FVIII inhibitors**	Every 4 weeks (6 mg/kg) (n=41)	4.5a	NR	1.4	74.1	

ABR: annualized bleeding rate; NR: not reported; a: Median ABR during the expansion phase.
*A bleed followed by treatment for a bleed (definition); bleeds due to surgery/procedures were excluded.

§ Oldenburg J, Mahlangu JN, Kim B, et al. *N Engl J Med*. 2017;377(9):809-818.
§§ Young G, Liesner R, Chang T, et al. *Blood*. 2019;134(24):2127-2138.
§§§ Mahlangu J, Oldenburg J, Paz-Priel I, et al. *N Engl J Med*. 2018;379(9):811-822.
§§§§ Pipe SW, Shima M, Lehle M, et al. *Lancet Haematol*. 2019;6(6): e295-e305.

Di Minno G. et al, *Blood Rev. 2022 Aug 23:101011.*

Short-term* and long-term** visits in PWH undergoing liver-directed gene therapy: rationale of biological fluid and clinical data collections.

see: Molecular therapy 4(6) (2001) 559-66: Molecular therapy 13(6) (2006) 1064-73, Blood 131(9) (2018) 1022-1031, New England journal of medicine 377(26): (2017) 2519-2530, Haemophilia 27(6) (2021) 947-956, and Gene Ther 2022; 29(1-2): 94–105 for details.

Blood/biological fluids to be collected	Aims, Comments
Blood: Monitoring clinical efficacy and safety of the treatment	Efficacy: Long-lasting factor activity and sustainable transgene expression Safety: AAV antibodies/neutralizing antibodies, anti-factor antibodies, inhibitors, liver enzymes, inflammatory markers, T-cell response.
Other biological fluids: Vector shedding analysis * **	Vector DNA is detectable for 2-28 weeks in urine, 16-52 weeks in feces, 4-52 weeks in saliva, and from 4 weeks to >1 year in blood.
Clinical information	
Bleeding events (numbers), Factor use (Units)§	To confirm sustainable transgene expression in the short- and long-lasting follow-up
Physical activity, Abstinence from alcohol	Limitations on physical exercise and abstinence from alcohol are requested to avoid muscle-related transaminitis elevations, especially in those that needed the use of corticosteroids
Contraception	Vector DNA is detectable for 4-56 weeks in semen. In animal studies, sperm cells are refractory to AAV transduction, thus reducing the risks of vertical transmission. Despite the limited risk of third-party infection, barrier contraception is recommended for up to 6-12 months.

***Weekly or up to tri-weekly visits (in the first 6 months); monthly or quarterly visits (up to 12 months)**
****Quarterly or bi-annual visits (for the remaining 4–5-year of follow- up)**
§Reported in patients' records and personal diaries
***** see text for details.**

Living with a hemophilia-free mind. §

Granting hemophilia-free days *			The feeling of being free from hemophilia *	
Basic assumptions in relation to available treatments§	**Daily worries addressed by PWH**		**Basic assumptions in relation to gene therapy§§**	**The new (old) dream of PWH**
Treatment schedule	(? My previous, my next treatment)		Achieving stable durable transgene expression	I feel the same as people without hemophilia. I do not think of hemophilia anymore.
Treatment efficacy	(? my bleeding risk today, able to do activities I like to do)			
Joint Pain	(? activities likely to cause less pain today)			
Coping strategies to use	(? coping by myself, needing help/support)			

* Modified from: *Krumb E, Hermans C., Res Pract Thromb Haemost. 2021;5: e12567*

§The current gold standard treatment of severe hemophilia is prophylaxis with IV infusions of EHL products once every 1 to 2 weeks for HB and twice a week for hemophilia A. Once every 1 to 2 weeks subcutaneous infusion of emicizumab is a valid alternative to EHL products in HA to grant increasingly higher numbers of hemophilia- free days.

§§ "Living with a hemophilia free mind" implies to achieve a stable durable transgene expression to lead to a new spirit in PWH.

Patient organizations and the process of education for the integration of gene therapy into the treatment armamentarium of people with hemophilia. °

Objectives	Key questions/phases of the process	Implications
Before gene therapy dosing		
-Raising awareness on gene therapy techniques and procedures, benefits, efficacy, durability, potential risks, and long-term follow-up requirements **-Communicating putative risks for informed consents ***	-What is important to the individual PWH in terms of treatment needs, measures of success, and potential risks and benefits? -Do PWH, families/doctors appreciate their key role for decision-making and their follow-up obligations?	Fostering objective straightforward discussions between gene therapy experts, PWH, their families and healthcare professionals
-Providing long-term information on benefits, efficacy, durability, and putative risks of gene therapy based on rAAV technology. **	-What is the evidence so far accumulated concerning measures of success (e.g., sustained efficacy) of the treatment in PWH that have undergone gene therapy -What is the evidence so far accumulated concerning potential risks (e.g., liver toxicity, inhibitor formation) in PWH that have undergone gene therapy - What is the evidence so far accumulated concerning success, potential risks, and unmet needs in PWH of strategies other than gene therapy	Supporting updated understandings of advantages and unmet needs in current treatments in hemophilia. §
-Supporting a continuous education and follow-up in gene therapy and an equal distribution of the ongoing experience at an international level. *** **Supporting an efficient network of hemophilia centers**	-Are all the centers worldwide aware of the experience gathered and the progress of PWH undergoing gene therapy? Consistent review	Assuring quick dissemination of new data across centers worldwide
Objectives	**Key questions/phases of the process**	**Implications**
After gene therapy dosing		
Strengthening positive relationships between hub and spoke centers******	-Are the two centers consistently reviewing the progress of each PWH? - Are the two centers identifying persons to contact for questions or concerns? -Do PWH and their families/caregivers receive appropriate psychosocial support after gene therapy dosing?	Confirming an active interaction between THCs with expertise in gene therapy (hub, treatment referral) and local THCs (spoke, screening and clinical follow-up).

° *For further details, see: Haemophilia: 26(4) (2020) 563-564, Haemophilia 27(4) (2021) 511-514.*

§Based on the evidence accumulated over the last 10-15 years.

* To ensure that PWH are fully aware of the risks and benefits of gene therapy prior to dosing, very clear pre-defined criteria should be pursued in clinical studies, and definite processes should be followed.

**Because of its clinical impact, such information should be communicated to patients with haemophilia (PWH); patient organisations and hemophilia doctors.

****Ad hoc* valuable resources will be provided by major International Institutions (the International Society on Thrombosis and Haemostasis [ISTH], World Federation of Hemophilia [WFH], European Association for Haemophilia and Allied Disorders [EAHAD], European Haemophilia Consortium [EHC], and American Thrombosis and Hemostasis Network [ATHN])

****Paper submitted

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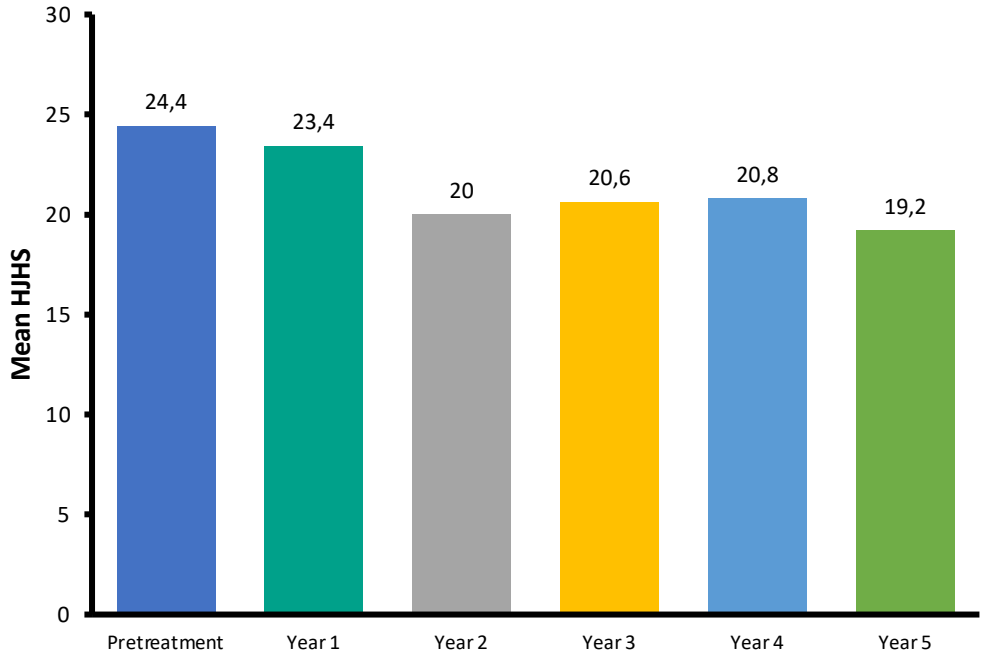
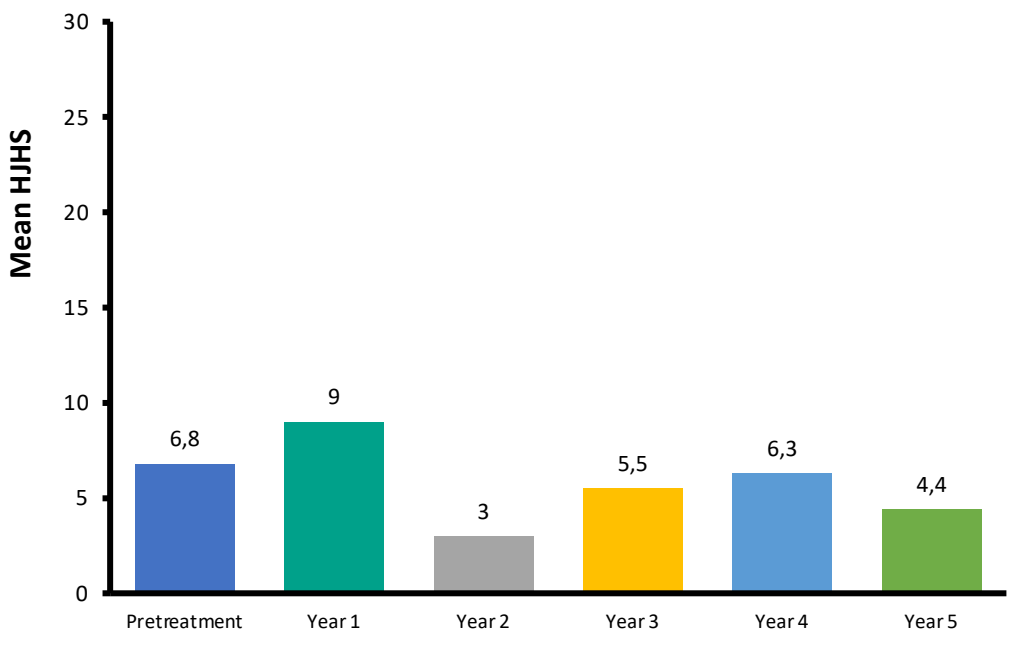
AMT-060 led to an improvement in joint health

AMT-060
Phase 1/2
5-yr data

Cohort 1



Cohort 2



Overall joint health improved when compared to pre-treatment HJHS scores

HJHS, Hemophilia Joint Health Score.

Miesbach W, et al. Oral presentation at ISTH congress, July 2021.

Phase 3

Additional endpoints for PwH

Efficacy[1,2]

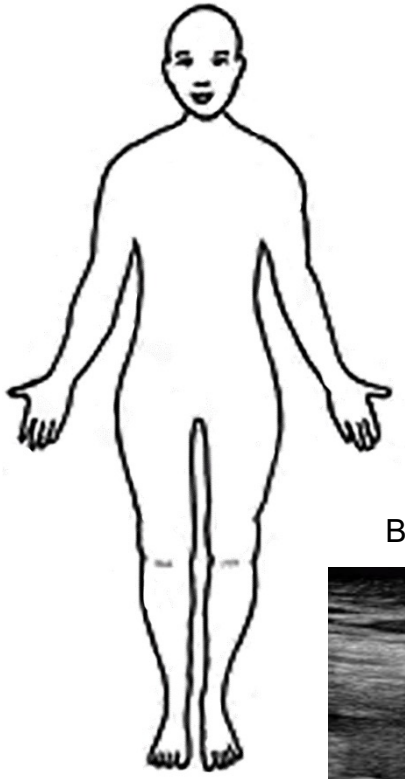
- Hemophilia Joint Health Score
- Assessment of joint health via musculoskeletal ultrasound
- PRO questionnaires: EuroQoL (EQ-5D-5L) and IPAQ
- Routine laboratory parameters

Safety secondary endpoints[1–3]

- Infusion site reactions
- Anti-AAV capsid-specific T cells
- Anti-FIX antibodies
- FIX inhibitor formation
- Vector DNA in blood and semen
- Inflammatory markers
- ALT/AST levels and corticosteroid use for AST/ALT increases
- Alpha-fetoprotein

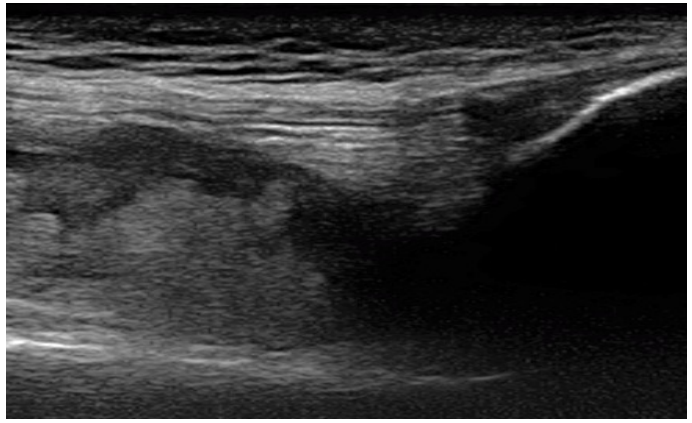
AAV, adeno-associated virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FIX, factor IX; IPAQ, International Physical Activity Questionnaire; PRO, patient-reported outcome. 1. EU Clinical Trials Register. EudraCT 2017-004305-40. Available at: https://www.clinicaltrialsregister.eu/ctr-search/search?query=2017-004305-40. Accessed August 2021. 2. ClinicalTrials.gov identifier: NCT03569891. Available at: https://clinicaltrials.gov/ct2/show/NCT03569891. Accessed August 2021. 3. Pipe SW, *et al.* Oral presentation at 62nd ASH meeting, December 2020.

Joint health data - knee

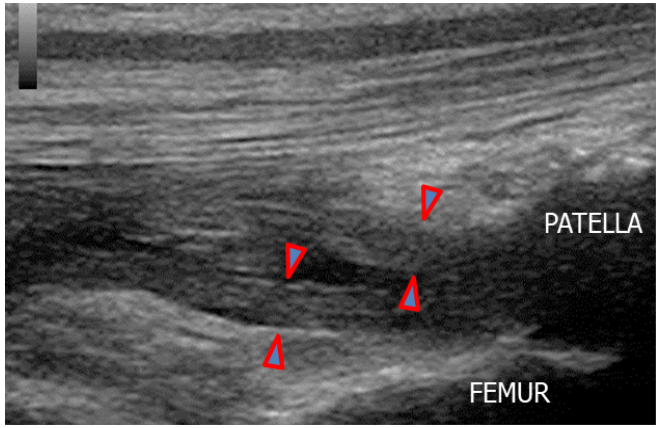


Right knee	
HJHS	4 (crepitus, flexion loss)
HEAD-US	4 (bone and cartilage damage) + 2 (synovitis)

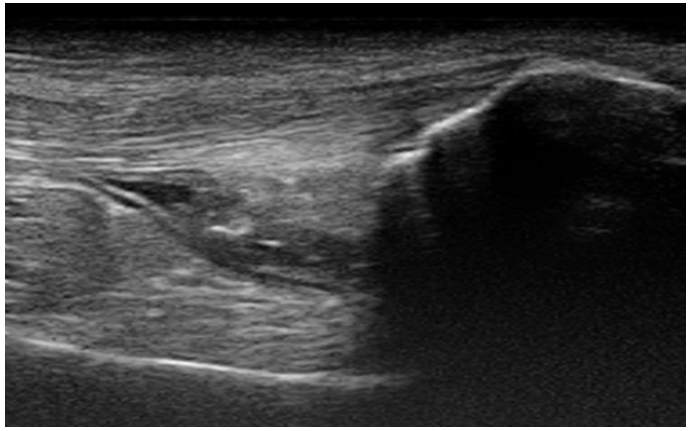
Baseline assessment



1st follow-up



2nd follow-up

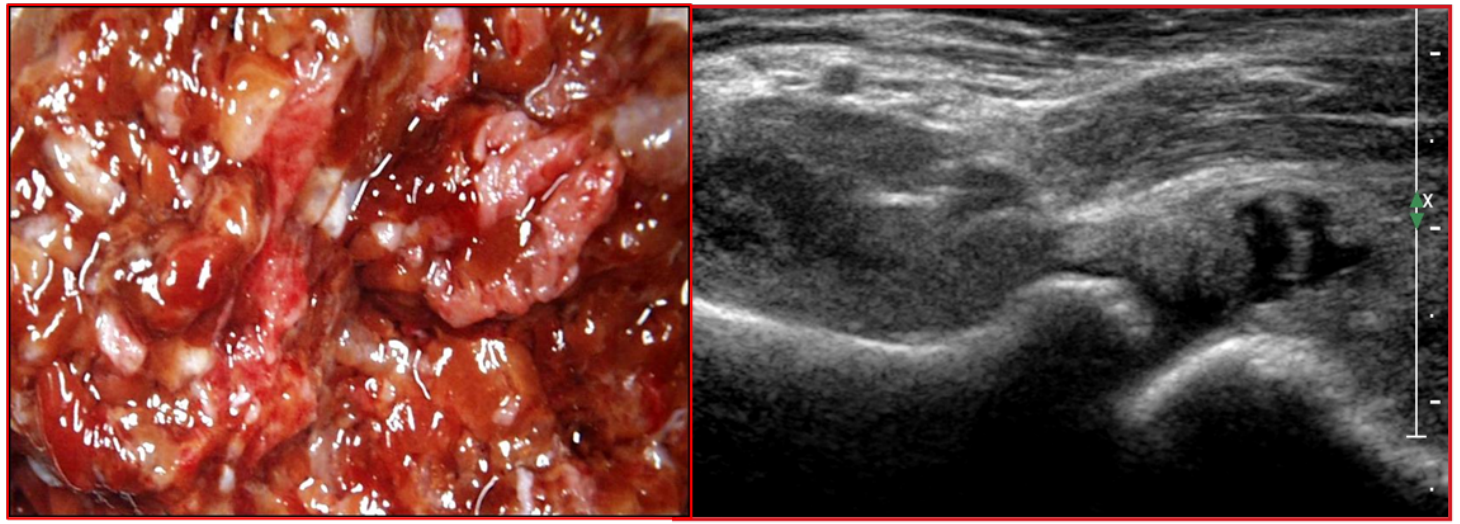


Synovitis reduction

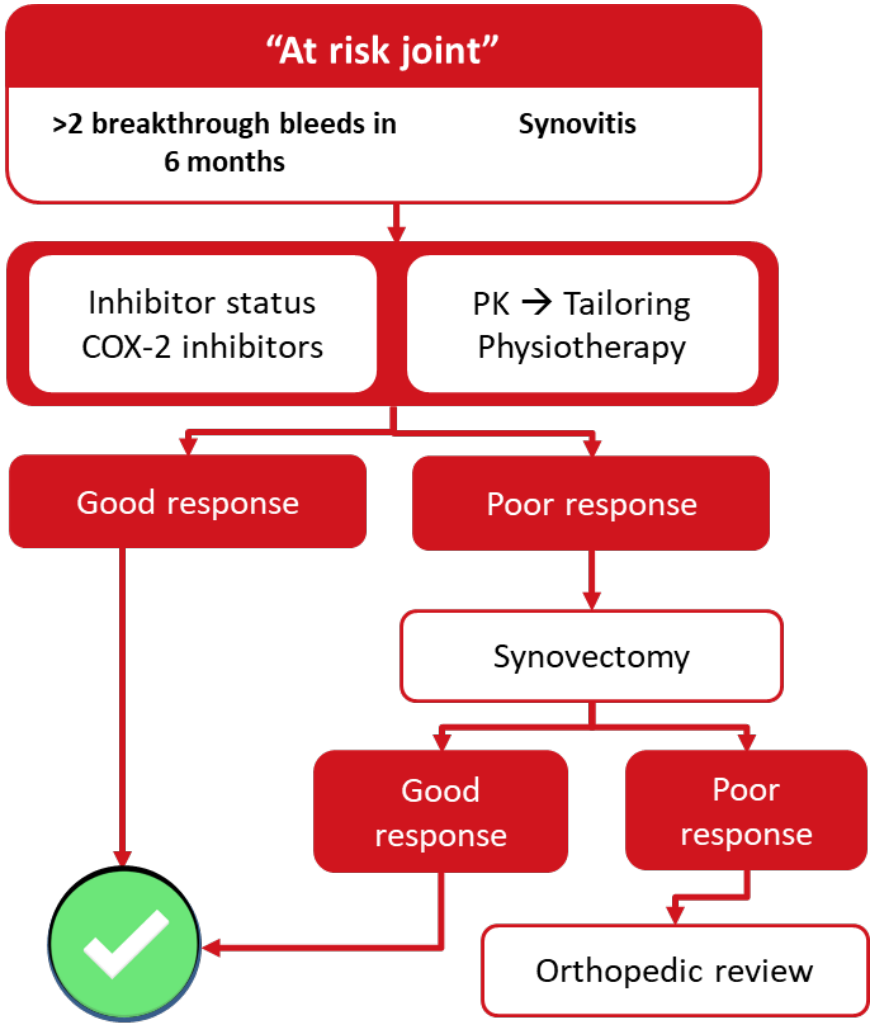
From “target joint” to “at risk joint”

Hypertrophic synovium ➡ Undertreatment

- Compliance problems
- Clinical phenotype variability
- PK variability
- High-risk daily activity



❖ Biomarker of previous (recent) joint bleeds



Di Minno MN et al, Br J Haematol. (2022);196(4):871-883; Hanley J. et al. Haemophilia (2017), 23, 511–520

Pharmacovigilance, prospective registries, and translational research to expand safe access to gene therapy in hemophilia.

Differential factor needs to prevent hemarthrosis in patients with or without target joints
Poor knowledge of factors influencing the development of transaminitis
Lack of expertise of most hemophilia doctors in managing/monitoring unexpected side effects in patients
Lack of expertise of most hemophilia doctors in central issues (e.g., eligibility criteria, predictability of response, duration of clinical benefit)
Defining variables (e.g., viral vector serotype/dose, manufacturing techniques) that control the integration ability of different vectors and/or the immune response against the rAAV capsid
Evaluations of patients' quality of life defined both as improvement in patient-relevant (e.g., chronic pain, mental health, infusion rates/year) and in patient-reported outcomes (e.g., use of healthcare resources, well-being, physical/mental health, physical activity, sports participation).
Providing the healthcare chain (PWH, health care professionals, patient associations, local, federal, and state government bodies, and reimbursement agencies) with updated information on: **✓ how gene therapy should be regarded as part of therapeutic options** **✓ (newer) laboratory methods to manage/monitor transgene expression in patients** **✓ cost-effectiveness analyses for a "one-time, one-dose cure".**

Networking!