Liver Biopsies – Molecular Analyses

Analysis of liver biopsies following AAV5-hFVIII-SQ administration revealed inter-individual variability in transgene mRNA and protein production

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Disclosures for Sylvia Fong

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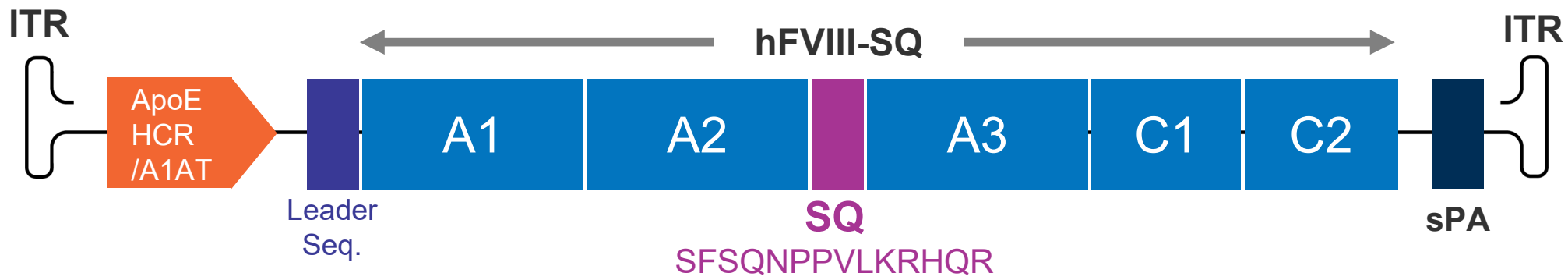
Valoctocogene Roxaparvovec (AAV5-hFVIII-SQ)



- AAV5 – single-stranded DNA
- Liver-specific promoter and synthetic polyadenylation signal
- Express a B-domain-deleted FVIII sequence (hFVIII-SQ)
- Amino acid sequence similar to existing recombinant FVIII therapy
- Produced in insect cells

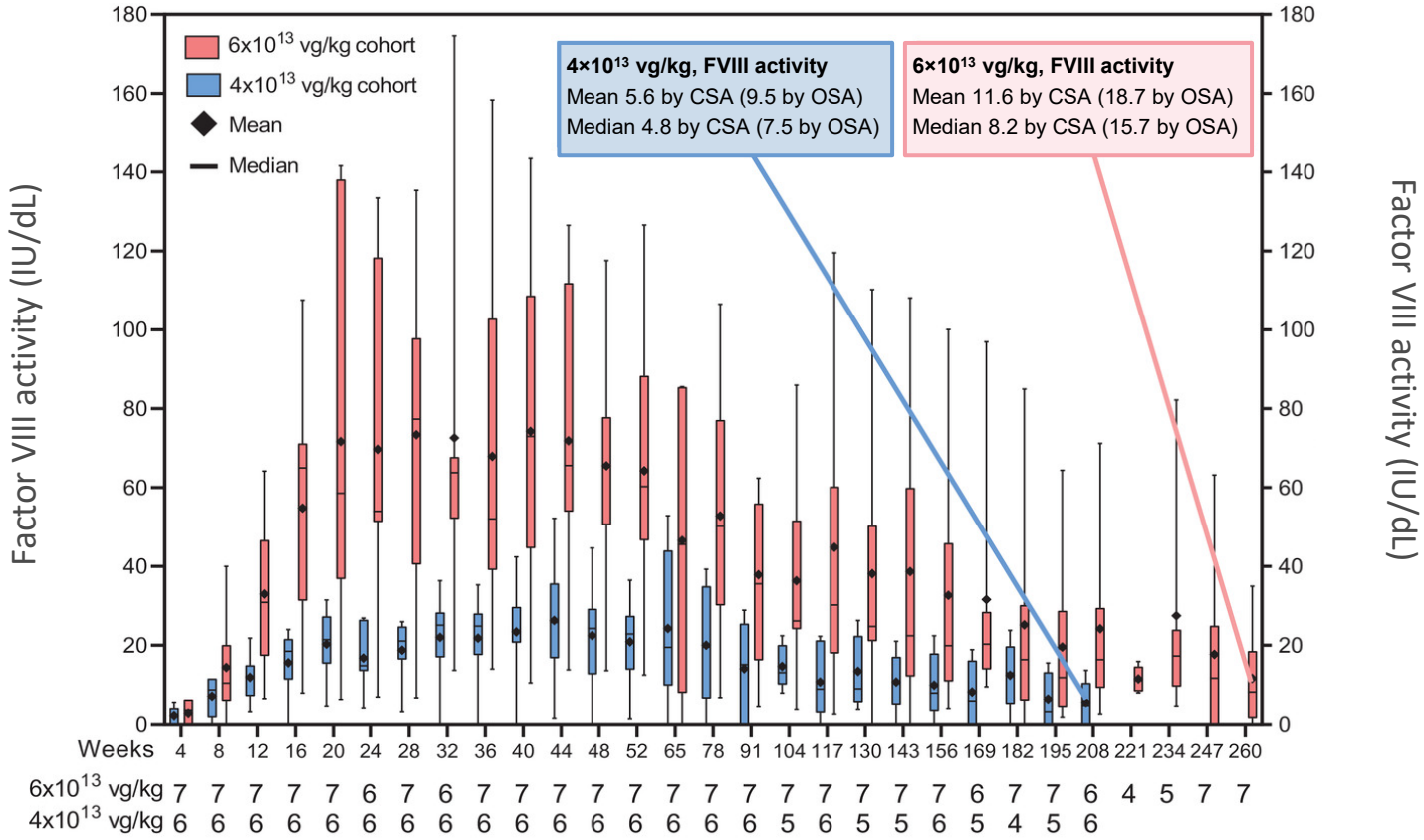
AAV5, adeno-associated virus serotype 5; hFVIII-SQ, B-domain-deleted human factor VIII sequence; ITR, inverted terminal repeat; sPA, synthetic polyadenylation signal

Bunting S, et al. Mol Ther 2018;26(2):496–509

Valoctocogene Roxaparvovec (AAV5-hFVIII-SQ)

- Efficacy in severe hemophilia A
  - 5-year expression[1]
  - Bleed reduction
  - Quality of life
- Safety
- Variability
  - Intra- and inter-study
- Durability

FVIII activity measured by the chromogenic substrate assay for participants in the 6 × 10^{13} and 4 × 10^{13} vg/kg cohorts over 5 and 4 years, respectively[a]



[a]FVIII activity levels taken within 72 h of exogenous FVIII administration were excluded.
FVIII activity that fell below the lower limit of quantitation (<3.0 IU/dl) was imputed as 0 IU/dl.
Whiskers represent the minimum and maximum values; boxes represent the 25th and 75th percentiles.

CSA, chromogenic substrate assay; FVIII, factor VIII; OSA, one-stage assay

1. Pasi KJ, et al. Haemophilia 2021;27(6):947–56

Liver biopsy substudy following AAV5-hFVIII-SQ

- An optional procedure offered to all subjects in the phase I/II trial
  - Optimally to include subjects across the different dose levels, with different FVIII activity level profiles, including subject with lowest FVIII activity level for cross-sectional sampling
  - Examination of histopathology of the liver, including possible safety findings with the caveat of lack of a baseline pre-gene therapy sample for comparison
  - Quantification of FVIII DNA, RNA, and protein expression in the liver to understand inter-subject variability
  - Determination of vector DNA forms relating to durability of expression

Key demographic and baseline clinical characteristics

Participants who underwent liver biopsy in the phase 1/2 clinical trial of valoctocogene roxaparvovec (AAV5-hFVIII-SQ)

Participant[a]	Dose of AAV5-hFVIII-SQ, vg/kg	Age at first enrollment[b], years	Biopsy timepoint (after first enrollment), weeks [years]	Biopsy date	Route of biopsy	No. of hepatic lobules in biopsy sample	ALT at time of biopsy, U/L	FVIII activity at time of biopsy, IU/dL[c]	
								CS result	OS result
1	6×10^{12}	25	201 [3.86]	Aug 2019	Transjugular	12	29	BLD	BLD
11	4×10^{13}	37	140 [2.69]	Aug 2019	Transjugular	23	11	18.6	28.4
15	4×10^{13}	37	148 [2.85]	Jan 2020	Percutaneous	15	20	BLD	2.1
3	6×10^{13}	32	214 [4.12]	Jan 2020	Percutaneous	23	12	8.2	14
4	6×10^{13}	23	213 [4.10]	Mar 2020	Percutaneous	18	11	13.5	23.9

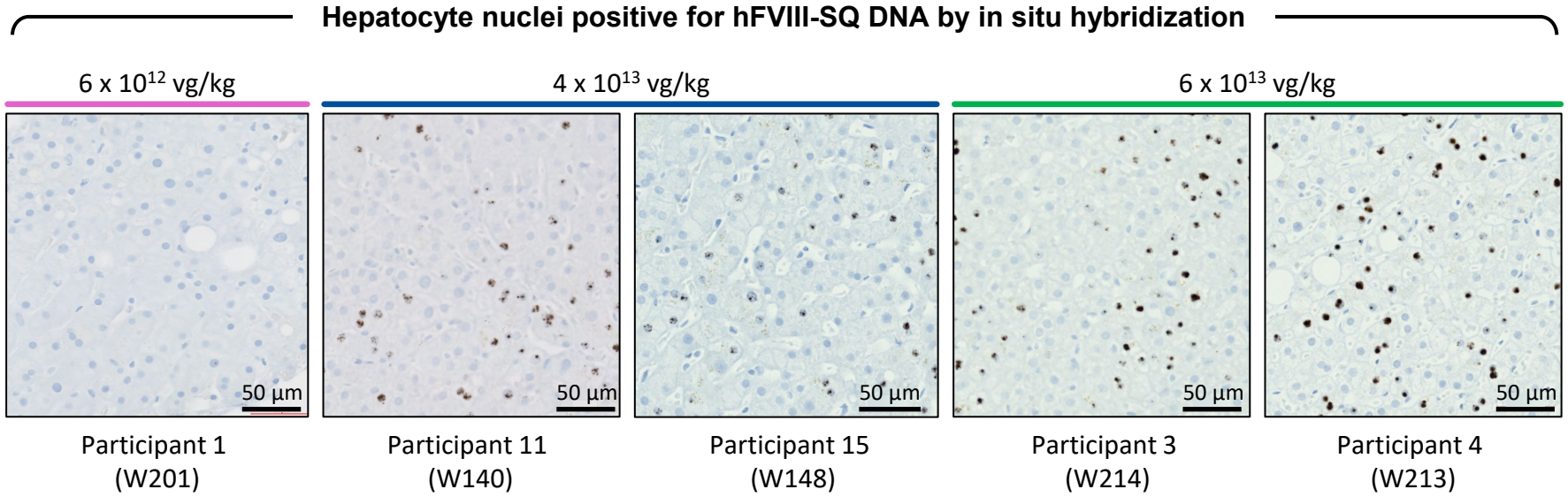
[a]Participants are numbered according to the order in which they were enrolled and dosed in the clinical trial.[1,2]
[b]Age at enrollment into the valoctocogene roxaparvovec phase 1/2 clinical trial (NCT02576795).
[c]FVIII levels were measured using both a OS activated partial thromboplastin time-based clotting assay and a CS assay.[3]

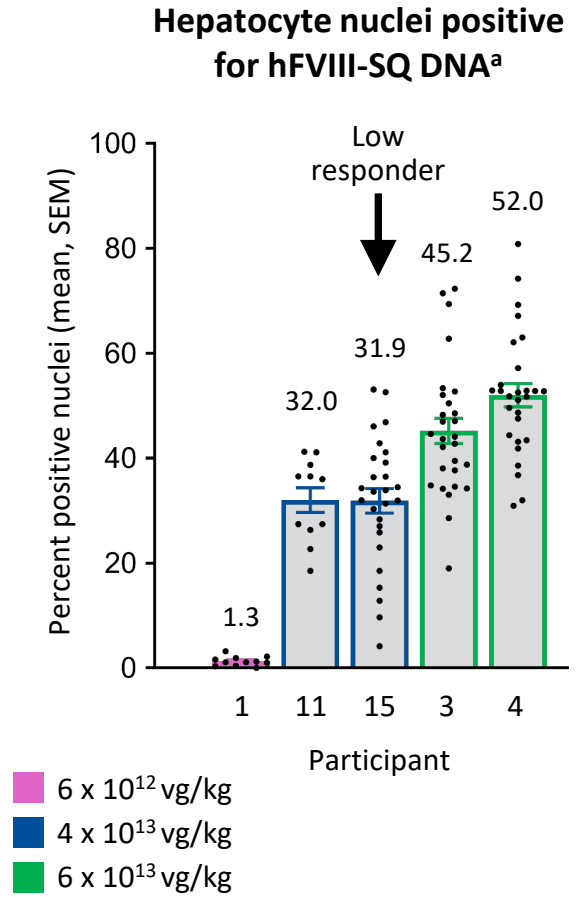
ALT, alanine amino transferase; BLD, below limit of detection; CS, chromogenic substrate assay; FVIII, Factor VIII; OS, one-stage clot assay; vg, vector genomes

1. Rangarajan S, et al. N Engl J Med 2017;377(26):2519–30
2. Pasi KJ, et al. N Engl J Med 2020;382(1):29–40
3. Rosen S, et al. Blood 2020;136(22):2524–34

AAV5-hFVIII-SQ transduction in human hepatocytes

- AAV5-hFVIII-SQ transduces human hepatocytes dose-dependently with vector genomes distributed in a panlobular pattern





[a]Data are means across 11 (participants 1 and 11) or 27–28 (participants 3, 4 and 15) images per biopsy section, spanning ≥50% of the tissue area (biopsy tissue area was larger for participants 3, 4 and 15). Dots represent quantification of each individual image; data labels show mean values.

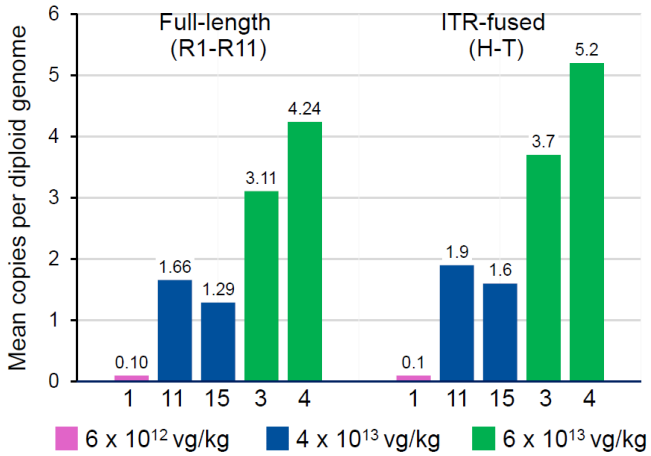
vg, vector genomes; W, week after dosing

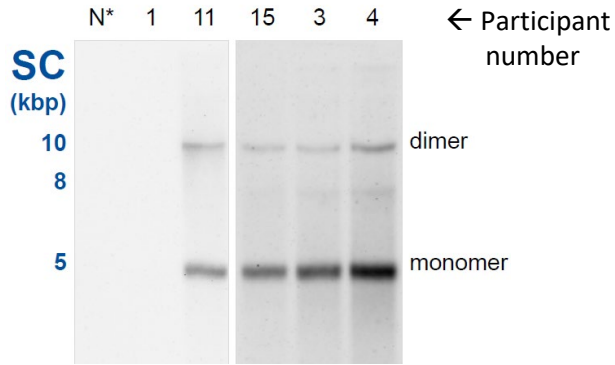
Fong S, et al. Nat Med 2022 (in press)

Vector genomes persist as full-length circular episomes

- Transduction levels appear to be similar for the non-responder (participant 15)
- Good levels of full-length circular DNA are still present **2.6 to 4.1 years after AAV5-hFVIII-SQ** administration

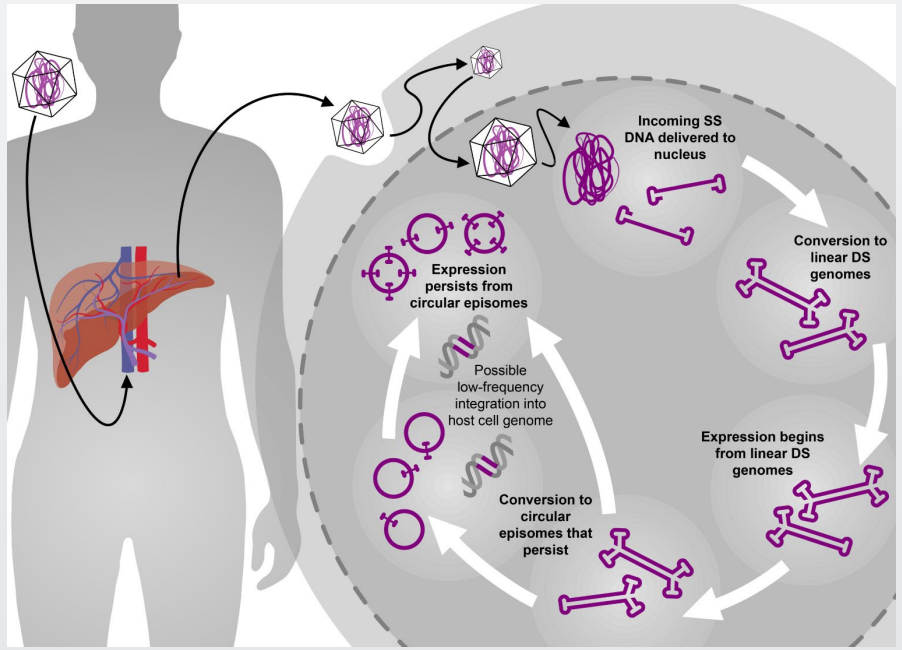
Circular genomes by drop-phase ddPCR





Qualitative Southern blot analysis of circular episomes

Liver-directed AAV vector administration, transduction of hepatocytes, and evolution of vector genome forms over time[1] Adapted from Wang et al.[2] and Sihn et al.[3]



*N, naïve

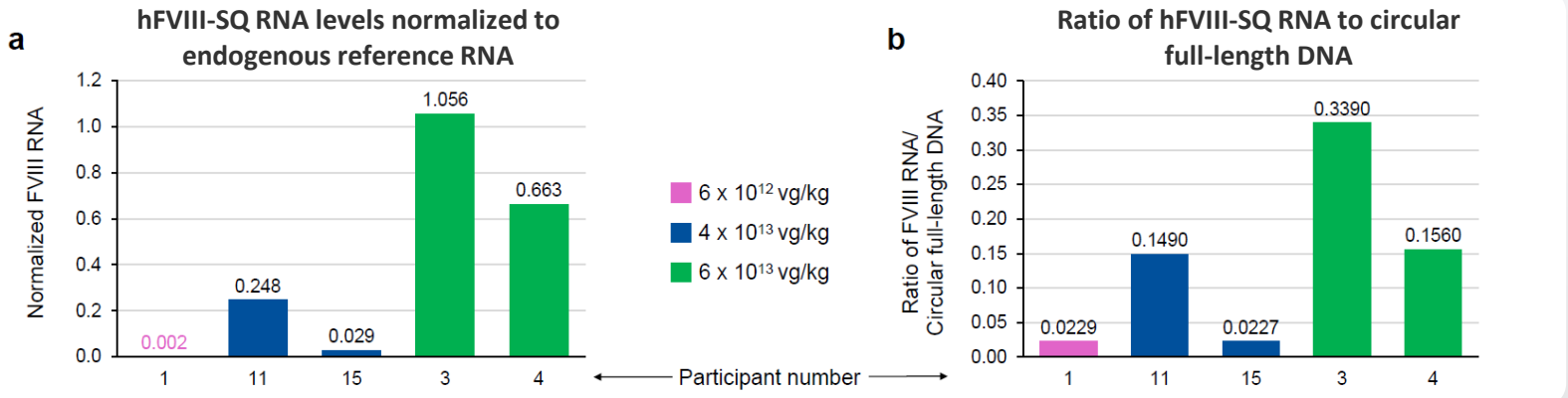
ddPCR, droplet digital PCR; kbp, kilobase pairs

1. Fong S, et al. Nat Med 2022 (in press)
2. Wang D, et al. Nat Rev Drug Discov 2019;18(5):358–78
3. Sihn CR, et al. Mol Ther Methods Clin Dev 2022;24:142–53

Low hFVIII-SQ transcript level detected in low responder

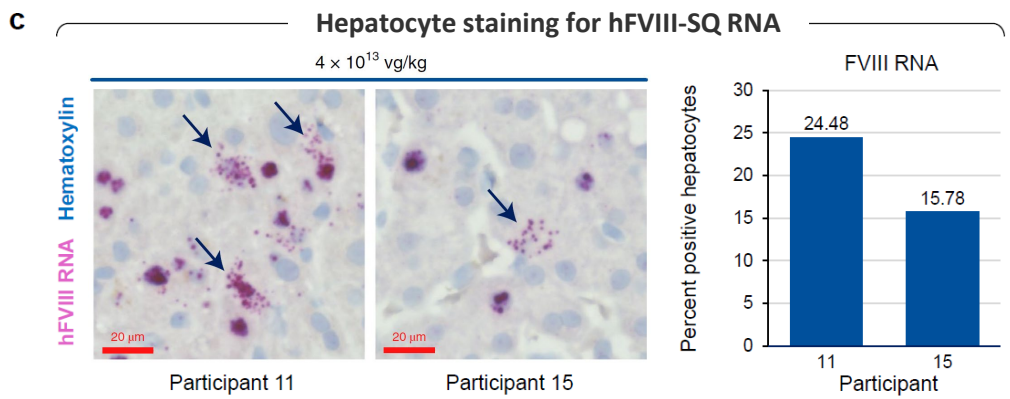
- In general, detected FVIII-SQ RNA levels were dose-dependent

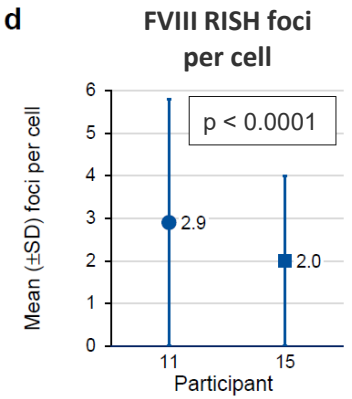
Detection of hFVIII-SQ transcript in adult liver biopsy samples by ddPCR

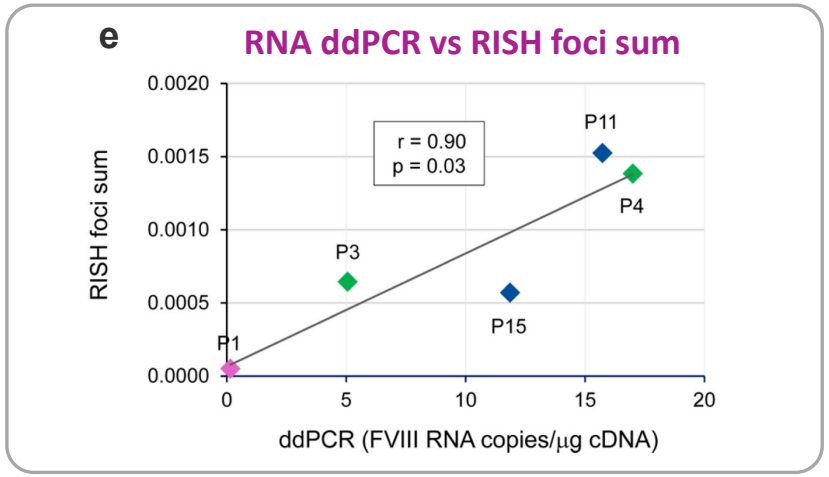


- In Participant 15, low FVIII-SQ RNA is a result of fewer hepatocytes expressing the transgene at a lower level per cell

Detection of hFVIII-SQ RNA in hepatocytes by ISH in 4 × 10^3 vg/kg dosed participants





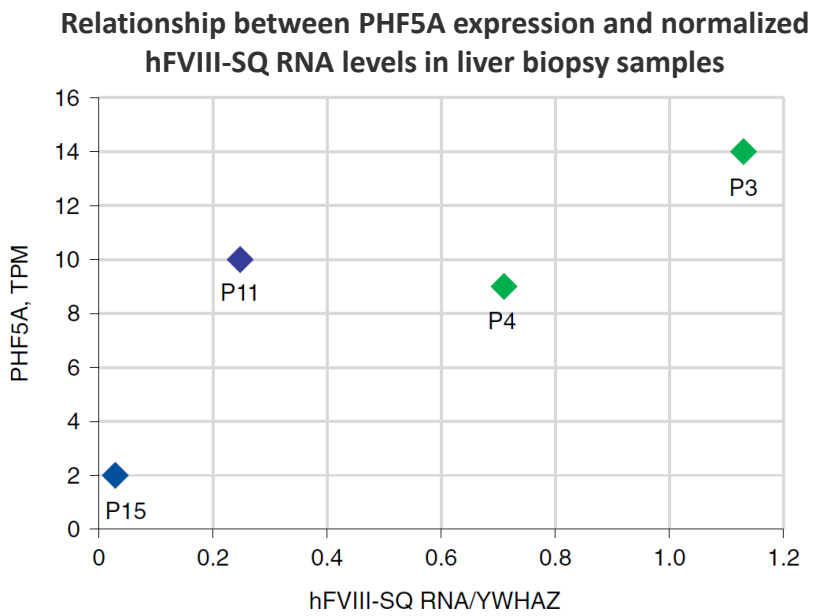


ddPCR, droplet digital PCR; ISH, in situ hybridization; RISH, RNA in situ hybridization; vg, vector genomes

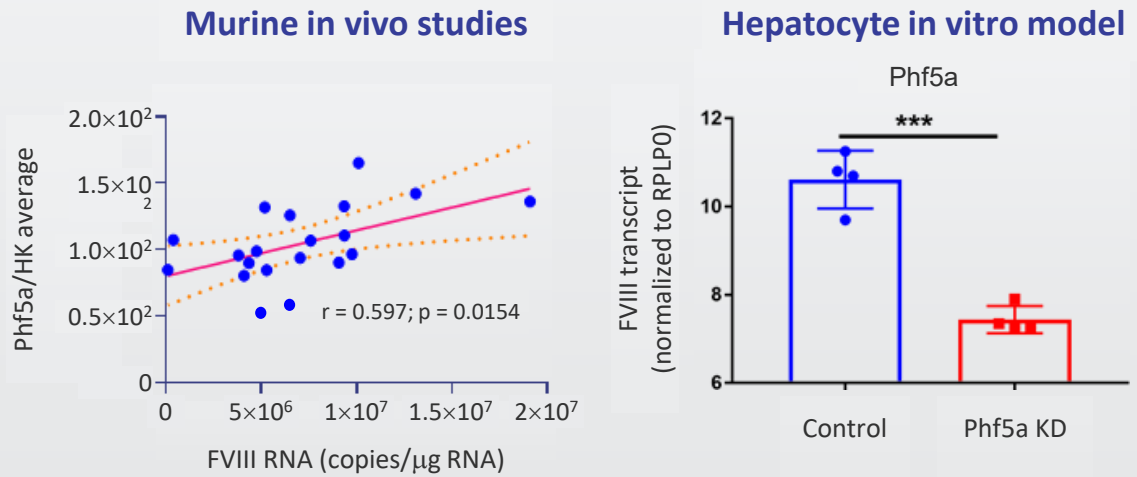
Fong S, et al. Nat Med 2022 (in press)

Molecules involved in positive regulation of transcriptional pathways were down-regulated in Participant 15

- **PHF5A** is involved in transcriptional elongation by RNA polymerase II and pre-mRNA splicing
- PHF5A was 5- to 7-fold lower in participant 15 compared to participants 3, 4 and 11[1]



- Nonclinical studies demonstrate association and mechanistic role between PHF5A and FVIII transcriptional activity[2]



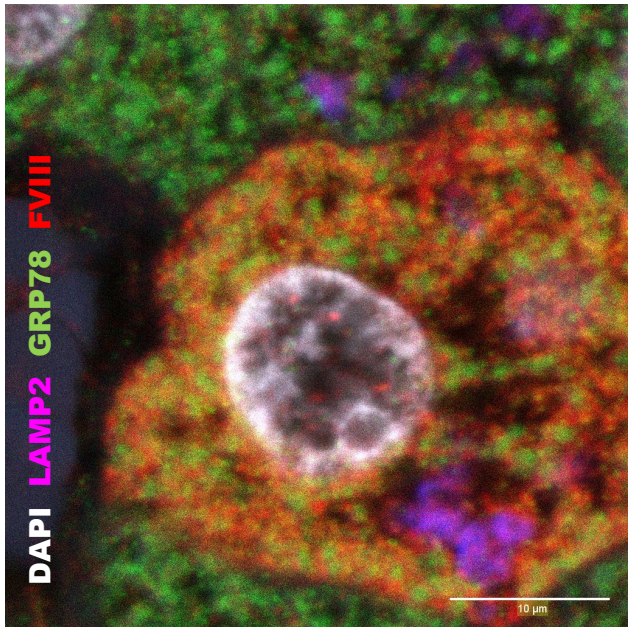
P, participant; PHF5A, plant homeodomain finger protein 5A; TPM, transcripts per million

1. Fong S, et al. Nat Med 2022 (in press)
2. Data on file, BioMarin Pharmaceutical Inc.

ER stress is not elevated in hepatocytes expressing hFVIII-SQ

- BDD FVIII-SQ protein is inefficiently folded and secreted from the ER
- Earlier data suggested that in vivo hydrodynamic delivery of plasmids that encode full-length or BDD-FVIII proteins may induce an **unfolded protein response**[1]
- More recent data in mice showed evidence of **ER stress response in animals** treated with a BDD-FVIII transgene with AAV8 vector or with a stronger promoter; **without any indication of hepatotoxicity**[2–4]

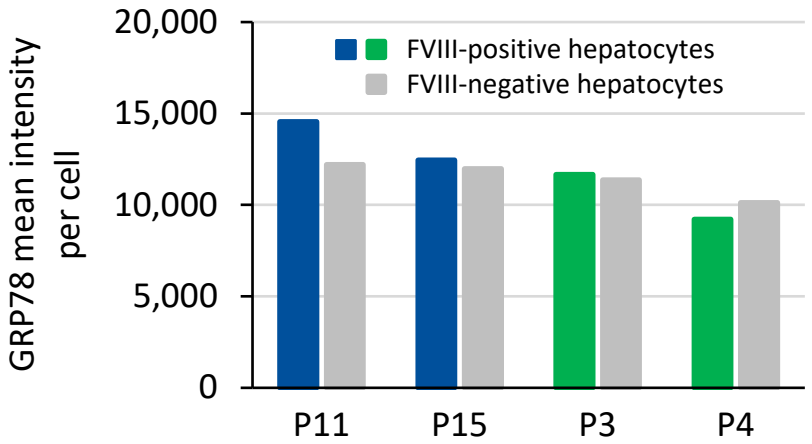
Grp78, an ER stress marker GRP78 is a key ER chaperone protein critical for protein quality control, interacts directly with hFVIII-SQ protein[5]; as well as controlling the activation of the ER-transmembrane signaling molecules



LAMP2 (lysosomal marker), **GRP78** (ER stress marker) **and hFVIII co-staining in one hepatocyte**[6]

- Shows FVIII staining both in the lysosome compartment (indicating FVIII being taken up via the endocytic pathway) and in the ER compartment (FVIII protein expressed from AAV5-hFVIII-SQ)

Quantitative analysis of GRP78 intensity per cell[6]



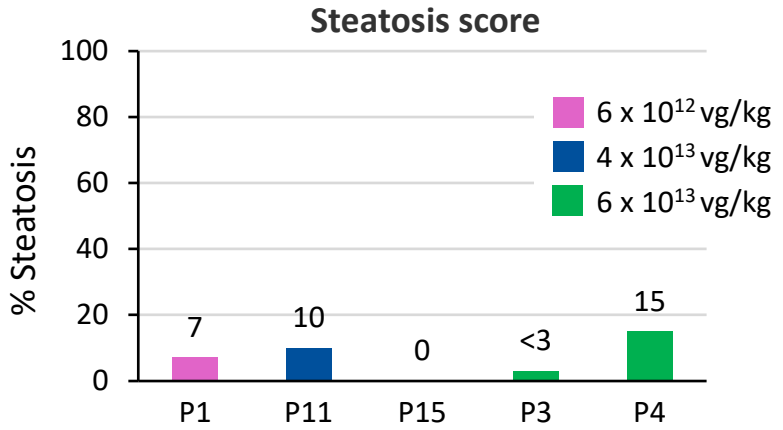
BDD-FVIII, B-domain-deleted factor VIII; ER, endoplasmic reticulum; P, participant

1. Malhotra JD, et al. PNAS 2008;105:18525–30; 2. Zolotukhin I, et al. Mol Ther Methods Clin Dev 2016;3:16063
3. Lange AM, et al. Mol Ther Methods Clin Dev 2016;3:16064; 4. Fong S, et al. Mol Ther Methods Clin Dev 2020;18:620–30
5. Poothong J, et al. Blood 2020;135(21):1899–911; 6. Fong S, et al. Nat Med 2022 (in press)

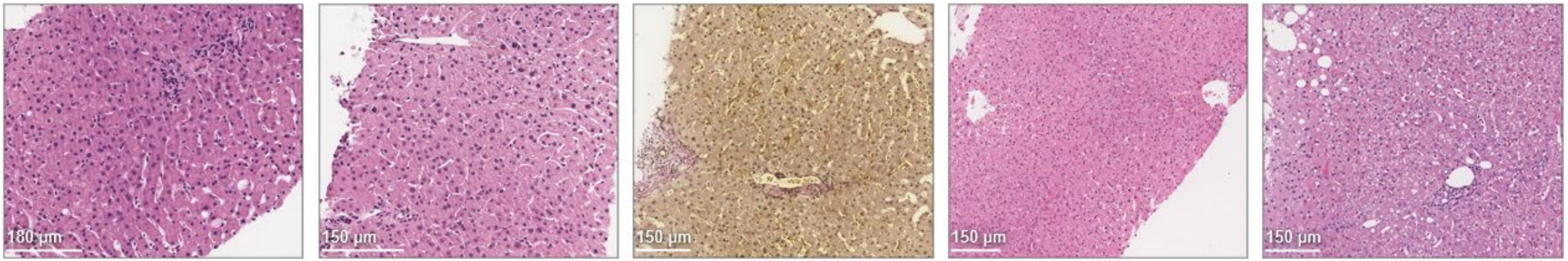
Histopathology

- No clinically relevant inflammation; sinusoidal infiltrates are common in liver biopsies without definitive chronic disease
- No significant fibrosis, and no evidence of dysplasia, necrosis or significant architectural distortion
- Mild steatosis in 4 out of 5 participants

Participant	Final pathological diagnosis
1	Mild steatosis with minimal changes
11	Minimal changes
15	No specific pathologic abnormality
3	Very mild steatosis with mild sinusoidal infiltrates with mild patchy mixed portal inflammation
4	Mild steatosis with minimal sinusoidal inflammatory infiltrates with mild patchy mixed portal inflammation



Histopathology sections stained with hematoxylin and eosin (Participants 1, 3, 4, and 11) or hematoxylin and Van Gieson (Participant 15)



Participant 1 | Participant 11 | Participant 15 | Participant 3 | Participant 4

P, participant

Fong S, et al. Nat Med 2022 (in press)

Analysis of human liver biopsies following AAV5-hFVIII-SQ administration

Key summary points

- Valoctocogene roxaparvovec (AAV5-hFVIII-SQ) transduces human hepatocytes dose-dependently with vector genomes distributed in a panlobular pattern
- Long-term expression of hFVIII following a single AAV5-hFVIII-SQ infusion was associated with the presence of durable genomes
- Inter-individual differences in transgene expression were noted despite similar successful transduction
  - May result from differences in expression of regulatory molecules involved in transcription and protein folding/secretion
- Histopathological examination of liver biopsies 2.6 to 4.1 years following AAV5-hFVIII-SQ showed
  - No evidence of endoplasmic reticulum stress in hepatocytes expressing hFVIII-SQ protein
  - No evidence of dysplasia, architectural distortion, fibrosis, or chronic inflammation

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Valoctocogene Roxaparvovec Study Participants

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