Early changes in liver transcriptomic profiles following adeno-associated viral gene therapy in the severe hemophilia A dog model

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Background

- Recombinant adeno-associated virus vector (rAAV)-mediated gene transfer is a promising therapeutic approach for genetic disorders such as hemophilia A, a recessive X-linked disorder characterized by excessive bleeding due to deficient or dysfunctional clotting factor VIII (FVIII) protein[1,2]
- Valoctocogene roxaparvovec (AAV5-HLP-hFVIII-SQ) is an AAV vector serotype 5 (AAV5) that delivers a B-domain–deleted FVIII coding sequence controlled by a liver-selective promoter[1,3]
  - In a phase 3 trial, valoctocogene roxaparvovec gene transfer reduced treated bleeding episodes for 2 years[3]
  - The most common adverse event, elevated alanine aminotransferase (ALT) levels, occurred in 88.8% of participants by 2 years post-infusion[3]
    - Delineating the mechanisms leading to transaminitis after rAAV-mediated gene transfer will enable advancement of this therapeutic approach
- A hemophilia A canine model can be used to study variable outcomes seen in clinical studies, factors influencing the expression of AAV5 constructs containing canine FVIII (cFVIII), early innate and adaptive immune responses, and cases of transaminitis

Aim

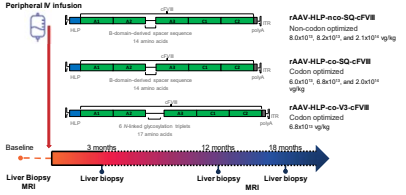
- To investigate liver gene expression profiles before and after AAV5-hybrid liver promoter (HLP)-canine-BDD-cFVIII administration in a severe hemophilia A dog model

Methods

Study design

- Nine dogs received a single infusion of 1 of 3 AAV5-HLP-cFVIII vectors at doses ranging from $6x10^{13}$ to $2x10^{14}$ vg/kg (**Figure 1**)
  - A liver magnetic resonance image (MRI) was taken at baseline and liver biopsies were taken at baseline and at 3 months post-dose
  - Further liver biopsies and MRIs are scheduled to take place at months 12 and 18
    - Safety biopsies are taken whenever ALT levels increased by a factor of ≥2
  - Blood samples were taken regularly for assessment of ALT, cFVIII activity, and immune responses

Figure 1. Study timeline and test constructs



cFVIII, canine factor VIII; co, codon optimized; HLP, hybrid liver promoter; ITR, inverted terminal repeat sequence; IV, intravenous; MRI, magnetic resonance imaging; nco, non-codon optimized; polyA, polyadenylation tail; rAAV, recombinant adeno-associated virus vector; SQ, B-domain deleted FVIII SQ variant.

Assays

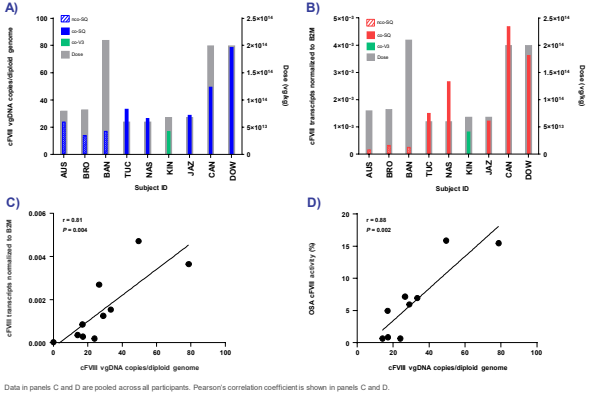
- Liver cFVIII DNA and RNA levels were measured using droplet digital PCR (QX ONE, QX2000; Bio-Rad, Hercules, CA)
- cFVIII activity was measured using an in-house one-stage FVIII assay using pooled canine plasma as a standard
- The cellular immune response in peripheral blood mononuclear cells was evaluated with an interferon gamma (IFN-γ) enzyme-linked immunosorbent spot assay (Mabtech, Nacka Strand, Sweden)
- Transcriptomic profiling was accomplished using RNA sequencing on liver biopsies with post-hoc pathway enrichment analysis
  - Reads were aligned to the *Canis familiaris* genome, and gene set enrichment analysis was performed using the ROSALIND platform (San Diego, CA)
- ALT levels were evaluated at a commercial veterinary reference laboratory

Results

Liver cFVIII expression

- Levels of vector genomes were dose dependent for codon optimized constructs at 3 months post-dosing (**Figure 2A**)
- Codon optimization enhanced vector DNA transcription levels at 3 months (**Figure 2B**)
- Vector genome DNA levels were strongly correlated with cFVIII transcript levels in data pooled across all samples (**Figure 2C**)
- cFVIII activity levels were strongly correlated with vector genome DNA levels at 3 months in data pooled across all samples (**Figure 2D**)

Figure 2. Transgene expression in liver biopsies at 3 months post-dose. A) Vector DNA. B) cFVIII RNA. C) Correlation of vector DNA and RNA. D) Correlation between vector DNA and cFVIII activity in plasma

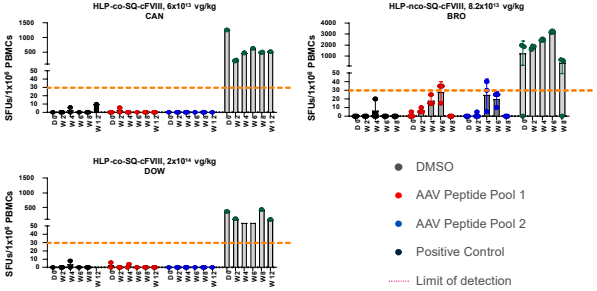


Data in panels C and D are pooled across all participants. Pearson's correlation coefficient is shown in panels C and D.
B2M, beta-2 microglobulin; cFVIII, canine factor VIII; co, codon optimized; nco, non-codon optimized; OSA, one-stage assay; vgDNA, vector genome DNA.

Immune responses

- No significant cellular immune responses were observed in peripheral blood mononuclear cells (PBMCs) after gene transfer (**Figure 3**)

Figure 3. Cellular immune responses over time



Three representative subjects are displayed.
AAV, adeno-associated virus; co, codon optimized; cFVIII, canine factor VIII; DMSO, dimethyl sulfoxide; HLP, hybrid liver promoter; nco, non-codon optimized; PBMC, peripheral blood mononuclear cell; SFU, spot-forming units.

Transcriptomics

- Several gene sets were enriched in the liver at 3 months compared to baseline in the data pooled across all samples (**Table 1**)
- Genes related to signaling receptor activity and cytokine receptor activity, growth factor activity and vascular endothelial growth factor receptor 3 binding, and leukocyte-endothelial cell adhesion and signal transduction were enriched at 3 months compared to baseline

Table 1. Gene sets enriched at 3 months vs baseline in the liver

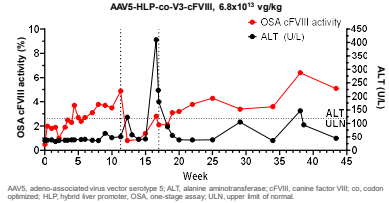
Enriched gene set	*P*-value	Adjusted *P*-value (Benjamini-Hochberg)	No. of genes in term	No. of genes that were regulated following treatment	No. of up-regulated genes	No. of down-regulated genes	Gene set NES
Integrin pathway	$5.8x1^{-4}$	0.034	22	3	3	0	1.47
B cells and plasmacytoid dendritic cells	$3.4x10^{-6}$	0.014	168	10	9	1	1.80
Common dendritic cells	$1.0x10^{-5}$	0.020	187	10	8	2	1.49
Cytokine production	$7.0x10^{-6}$	0.015	668	19	16	3	1.56

Gene set NES determines whether a gene set is upregulated or downregulated. An NES with a Benjamini-Hochberg adjusted $P < 0.05$ was considered statistically significant.
NES, normalized enrichment score.

ALT elevations

- One dog experienced 2 ALT elevations (**Figure 4**)
  - First elevation directly followed a planned liver biopsy, and was therefore likely to be related to the procedure
  - Second elevation was characteristic of transaminitis cases observed in clinical trials, and a biopsy was taken for further analysis

Figure 4. Episodes of transaminitis and transient loss of FVIII expression in a dog treated with $6x10^{13}$ HLP-co-V3-cFVIII



AAV5, adeno-associated virus vector serotype 5; ALT, alanine aminotransferase; cFVIII, canine factor VIII; co, codon optimized; HLP, hybrid liver promoter; OSA, one-stage assay; ULN, upper limit of normal.

Mechanisms of ALT elevations

- Glucose-regulated protein (GRP78) expression did not increase in hepatocytes expressing cFVIII, indicating no ER stress (data not shown)
- Gene ontology annotations comparing baseline vs ALT rise (week 17) RNAseq data revealed bacterial exposure and gene signatures relevant to immune clearance of protozoan or bacterial infection (data not shown)

Conclusions

- Transcriptomic profiling of canine liver samples indicated that mild activation of B cells, dendritic cells, NK-cell and T-cells with an inflammatory cytokine responses occurred in the liver of AAV5-HLP-cFVIII treated dogs 3 months post gene transfer
- We are using both human (AAV5-HLP-hFVIII-SQ–administered) and canine (AAV5-HLP-cFVIII–administered) biopsies to investigate the mechanisms of ALT elevation
- Transcriptomic profiling of PBMCs is ongoing and will be compared with liver results to better understand the kinetics of rAAV-induced immune responses

References

1. Bunting S, et al. *Mol Ther.* 2018;26:496-509. **2.** Srivastava A, et al. *Haemophilia.* 2020;26:1-158. **3.** Mahlangu J, et al. *N Engl J Med.* 2023;388:694-705.

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Disclosures

AMI, **BY**, **NAK**, **LR**, and **SF** are employees and shareholders of BioMarin Pharmaceutical Inc. **PB** has received research support from, and acted in an advisory role for, BioMarin Pharmaceutical Inc. **DL** has received research support rom CSL Behring and BioMarin Pharmaceutical Inc. **A Mo**, **LH**, **A Menard**, **AP**, and **AW** have no conflicts to declare.

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