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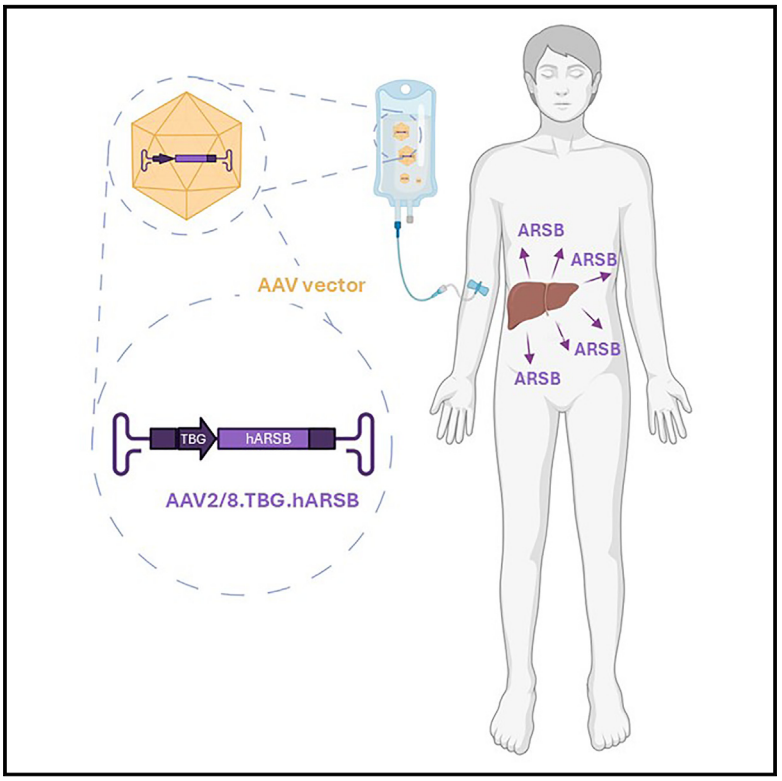
Multi-year enzyme expression in patients with mucopolysaccharidosis type VI after liver-directed gene therapy

Graphical abstract



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In brief

Rossi et al. presented longitudinal data on mucopolysaccharidosis type VI individuals who discontinued enzyme replacement therapy and received AAV-mediated liver-directed gene therapy. Sustained serum ARSB expression and a modest increase in urine glycosaminoglycans were observed. No significant changes were detected in endurance, or pulmonary and cardiac function.

Highlights

- Long-term data of AAV-mediated liver-directed gene therapy in patients with MPS VI
- Sustained ARSB expression and modest increase in urine glycosaminoglycans
- Endurance, pulmonary and cardiac function showed no changes despite ERT interruption

Translation to Patients

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Multi-year enzyme expression in patients with mucopolysaccharidosis type VI after liver-directed gene therapy

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CONTEXT AND SIGNIFICANCE Mucopolysaccharidosis type VI (MPS VI) is a rare lysosomal storage disease due to arylsulfatase B (ARSB) deficiency that leads to a multisystem accumulation of glycosaminoglycans (GAGs). Current treatment based on weekly infusions of enzyme replacement therapy (ERT) is unsatisfactory. In this study, after the discontinuation of ERT, MPS VI individuals received a single intravenous infusion of an AAV vector that delivered the gene encoding ARSB to the liver. AAV vector administration was safe and resulted in sustained ARSB expression for at least 3 years post-injection. Although a modest increase in urine GAGs was detected, no significant changes in endurance, liver and spleen sizes, or pulmonary and cardiac functions were observed. These findings support the safety and efficacy of AAV-mediated liver-directed gene therapy for MPS VI.

SUMMARY

Background: Mucopolysaccharidosis type VI (MPS VI) is due to a deficiency of the lysosomal enzyme arylsulfatase B (ARSB) that results in multi-organ accumulation of glycosaminoglycans (GAGs). Limitations of current treatments prompted the development of a liver-directed gene therapy clinical trial for MPS VI.
Methods: We report the long-term follow-up of patients with MPS VI who discontinued enzyme replacement therapy (ERT) and received a single intravenous infusion of high-dose (6×10^{12} genome copies/kg) recombinant adeno-associated virus serotype 8 (AAV8) vector expressing ARSB under the control of a liver-specific promoter (ClinicalTrials.gov: NCT03173521). Primary outcomes were safety and urinary GAG excretion. Secondary outcomes were endurance and respiratory function.

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Findings: Median follow-up time was 45 months (n = 4, three females and one male; age range: 5–10 years). No late-emergent safety events were observed. Patients showed sustained serum ARSB activity (38%–67% of mean healthy reference values), a modest increase in urinary GAG concentrations, and no relevant changes in endurance, cardiac, or pulmonary function. In one of the four patients, ERT was restarted because of elevated urinary GAGs without decreased serum ARSB activity up to about 2.5 years after gene transfer. Liver and spleen size remained within the reference ranges.

Conclusions: A single intravenous administration of AAV8.TBG.hARSB was safe and resulted in sustained ARSB expression and a modest increase in urinary GAGs in most patients, thus supporting liver-directed gene therapy for MPS VI.

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INTRODUCTION

Liver-directed gene therapy based on adeno-associated virus (AAV) vectors is being investigated in clinical studies of a growing number of inherited metabolic disorders.[1] Gene therapy aims to induce high levels of enzyme activity in multiple tissues, either by engrafting gene-modified hematopoietic stem progenitor cells (*ex vivo*) or by direct infusion of a viral vector expressing the therapeutic gene (*in vivo*). Both modalities have been investigated in lysosomal storage disorders.[2] Maroteaux-Lamy syndrome, or mucopolysaccharidosis type VI (MPS VI), is an autosomal recessive lysosomal storage disease with an incidence of 0.04–0.43 in 100,000 live births.[3] MPS VI is due to arylsulfatase B (ARSB) deficiency that leads to multisystem accumulation of glycosaminoglycans (GAGs) in several tissues. Enzyme replacement therapy (ERT) consisting of weekly intravenous infusions of recombinant enzyme and hematopoietic stem cell transplantation (HSCT) are available treatments for several lysosomal storage disorders, including MPS VI.[4] In patients with various types of mucopolysaccharidoses, including MPS VI, decreased liver and spleen sizes, stabilization or improvement in endurance and pulmonary function, and reduction in urinary GAG excretion have been observed after starting ERT.[5–7] Following infusion, the enzyme reaches maximum blood concentrations between 120 and 240 min but is rapidly cleared from the circulation thereafter and becomes undetectable within 10 min after completion of the enzyme infusion.[8] Available treatments have disadvantages: ERT requires life-long and costly parenteral infusions with considerable inconvenience for patients and their caregivers, whereas allogeneic HSCT is hampered by graft failure, limited availability of human leukocyte-antigen-matched donors, and significant hazards related to preconditioning regimens and graft-versus-host disease.[4]

Preclinical studies in small and large animal models of MPS VI have shown that a single intravenous administration of an AAV2/8 encoding the ARSB under the transcriptional control of the liver-specific thyroxine-binding globulin (TBG) promoter resulted in sustained ARSB expression, reduced tissue and urinary concentrations of GAG, and improved motor activity and bone growth.[9–13] These results highlighted the potential of gene therapy to overcome the limitations of currently available treatments and prompted the development of this phase 1/2 clinical study in MPS VI. A single intravenous administration of an AAV2/8 encoding ARSB had no dose-limiting side effects or adverse event profiles.[14] In this article, we present the long-term results of the four patients with MPS VI treated with a high dose of AAV over a maximum follow-up of 4 years.

RESULTS

Four patients with MPS VI (one male and three females) were enrolled in the high-dose (6×10^{12} genome copies [gc]/kg) cohort. The demographics and genotypes of these individuals were previously reported[14] and are summarized in Table S1. Here, we present safety and efficacy data obtained at least 3 years after administration of the gene therapy vector in all four participants (subjects 006–009). The median follow-up period after gene therapy was 45 months (195 weeks) (min-max = 36-48). The ages of these four patients at treatment ranged from 5 to 10 years, with a mean of 8.75 years (SD = 2.50). Baseline serum ARSB was low or undetectable in all four, with participant 01-008 showing higher residual serum ARSB activity. No late emergent safety events were recorded in any of the patients, including cancer, autoimmune disorders, or liver disease. Neither increased serum α-fetoprotein (AFP) nor liver masses were detected throughout the follow-up period. Apart from the two previously reported serious adverse events (SAEs) that occurred in one patient (participant 006), one subsequent SAE was recorded in one patient (participant 009) at 24 months (104 weeks) of follow-up. The complete list of additional SAEs and AEs is presented in Table 1. None of the events was deemed by the investigators to be related to the study drug. No clinically significant rise in aspartate (AST) or alanine (ALT) transaminase activities were detected after vector administration[14] or in the measurements performed every 6–12 months. Because of worsening of pre-existing hip dysplasia, participant 009 underwent elective hip surgery without complications between weeks 69 and 91 after gene therapy.

After infusion of the vector, all patients showed sustained ARSB activity (006: 1,235 pg/mL, 007: 799 pg/mL, 008: 991 pg/mL, and 009: 1,422 pg/mL at the latest measurements) that corresponded to 38%–67% of mean values in the healthy population (i.e., 2,119 pg/mL) (Figure 1A). In contrast to patients who received the low (6×10^{11} gc/kg) or intermediate (2×10^{12} gc/kg) doses and returned to ERT, only a modest increase in urinary GAGs was observed in patients receiving the high dose (6×10^{12} gc/kg). Nevertheless, the increases in urinary GAGs were below the average concentration of untreated patients with

Table 1. Serious adverse events and adverse events in the study participants enrolled in the high-dose cohort

Type	Grade	No. of events	Participant ID
SAEs			
Upper airway infection and nosebleed	3	1	009
AEs			
Aortic root dilation[a]	2	1	006
Upper respiratory infection	2	1	009
SARS-CoV-2 infection	1	2	006
Unilateral transmissive hearing loss (right ear)[a]	1	1	006
Worsening of vision[a]	1	1	008
Hepatomegaly[a]	1	1	008
Fluctuant mobile mass in left abdomen	1	1	008
Rash and fever	1	1	008

SAEs and AEs recorded after the previous analysis[14] are reported. The severity of adverse events was determined according to the Common Terminology Criteria for Adverse Events v.5. None of the events were deemed by the investigators to be related to the study drug. SAEs, serious adverse effects; AEs, adverse effects.
[a]AEs likely due to progression of the underlying condition.

MPS VI (321 mg/g creatinine) (Figure 1B). Three out of four patients remained without ERT for up to 48 months (208 weeks) (range: 42–48 months, 182–208 weeks) after vector infusion. One patient (participant 008) showed increased urinary GAG excretion at 2.5 years after gene therapy, approaching the threshold of untreated patients. Although neither a reduction in serum ARSB nor clinical worsening was observed, a decision was taken to restart ERT. At the last follow-up, total anti-ARSB antibodies were below the limit of detection in participants 006 and 009 and clearly measurable in participant 007. In participant 008, total anti-ARSB antibodies became measurable after resuming ERT (Table S2).

No statistically significant changes were observed in the median endurance evaluated by the 6 min walking test (6MWT) (467 vs. 535 m, $p = 0.125$) and the 3 min stair climb test (3MSCT) (1.38 vs. 1.51 steps/s, $p = 0.625$) at last follow-up as compared to baseline (Figures 2A and 2B). Participants 007 and 009 showed improved 6MWTs from baseline (+18% and +23%, respectively) and 3MSCTs (+32% and +34%). Although two patients showed mildly reduced forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC), even in the absence of respiratory symptoms, the median values at the last follow-up did not vary significantly compared to the baseline (FEV_1: 80.85% vs. 62.65%, $p = 0.125$; FVC: 78.05% vs. 64.15%, $p = 0.250$) (Figures 2C and 2D; Table S3). Liver and spleen size did not change significantly at last follow-up compared to baseline ($p = 1.000$ and $p = 0.750$, respectively) and remained within normal reference ranges, albeit one participant showed a marked increase in the centile of the spleen size (Table 2). The median interventricular septum (IVS) thickness (Z score: 0.50 vs. 0.49, $p = 0.857$), left ventricular end-diastolic diameter (LVEDD) (Z score: −0.10 vs. 0.39, $p = 1.000$), and left ventricle ejection fraction (59.50% vs. 60.00%, $p = 1.000$) remained unchanged at last follow-up compared with baseline. Aortic root dimension showed no significant changes in three subjects (006, 007, and 008). In one subject, a mild increase was observed (009; the Z score of the aortic root increased from +2.15 to +2.56), but the moderate aortic valve regurgitation remained unchanged. Cardiac valvular disease was stable in two subjects (008 and 009), while mild worsening occurred in two subjects (from mild to moderate mitral valve regurgitation in 006 and

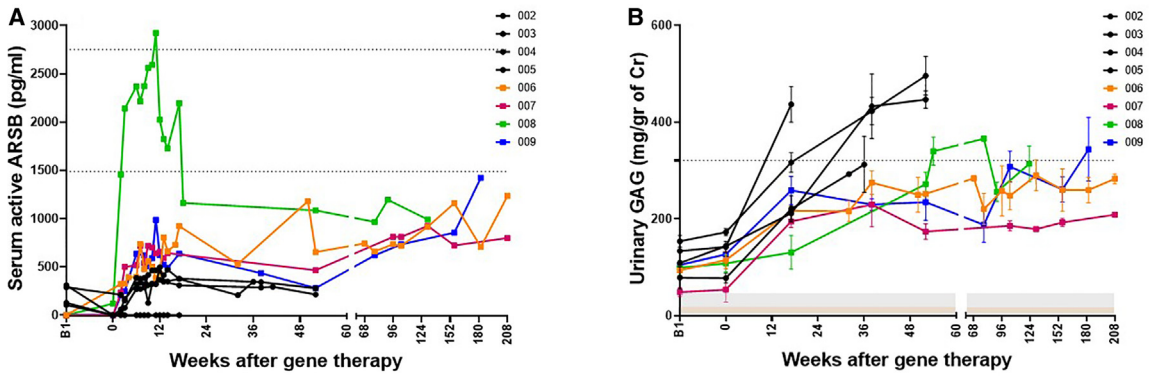

Figure 1. Serum active ARSB and urinary GAGs

(A) Serum active arylsulfatase B (ARSB) before and after gene therapy in participants who received the low and intermediate dose (black circles) and the high-dose vector (colored squares). Serum active ARSB was determined by comparing the enzymatic activity of the test samples to that of a standard curve made with recombinant human ARSB. The reference activity range measured in healthy individuals (i.e., 2,119 ± 632 pg/mL) is displayed by dotted lines at y = 1,487 and y = 2,751. (B) Urinary glycosaminoglycan (GAG) excretion before and after gene therapy in participants enrolled in the low and intermediate dose (black circles) and the high-dose cohorts (colored squares). Each value is the mean (±standard error) of urinary GAGs measured in two or three samples collected over 2 or 3 consecutive days. For participant 008, only the values measured before restarting enzyme replacement therapy (ERT) are shown in the graph. The gray shaded area represents the reference range of urinary GAGs in healthy individuals aged 5–13 years. The pink shaded area represents the reference range of urinary GAGs in healthy individuals aged 13 years and older. The dotted line displays the threshold to restart ERT. Patient identification numbers are shown in the key.

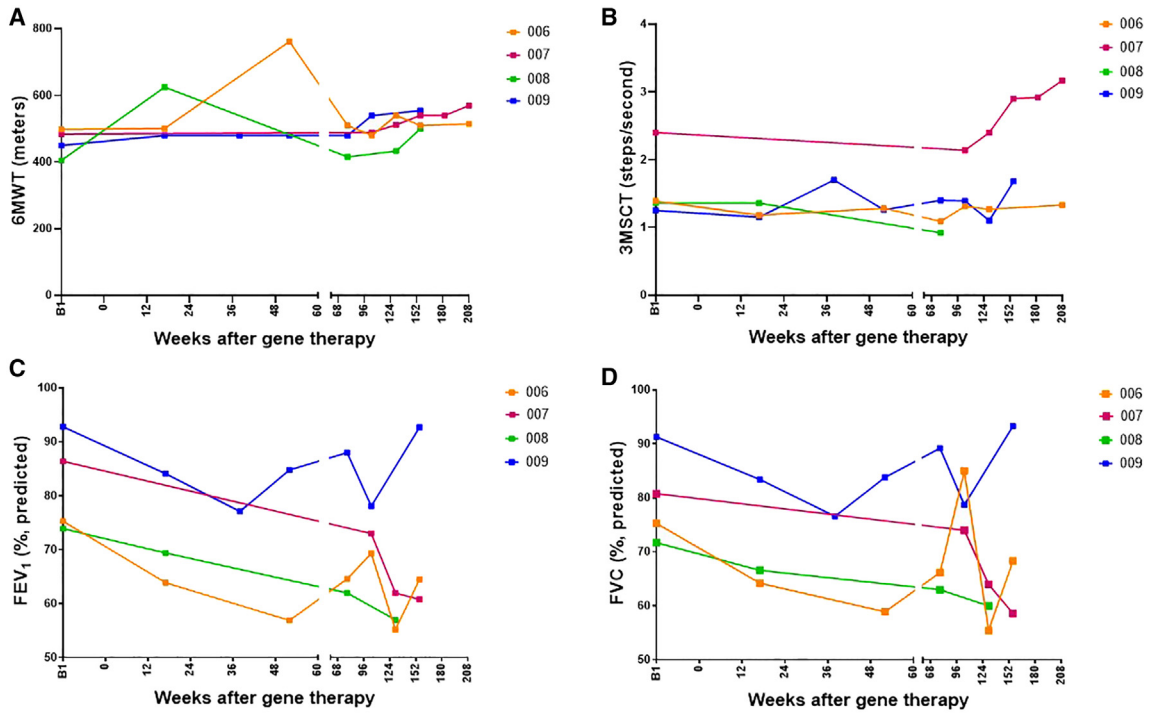

Figure 2. Clinical outcomes in study participants enrolled in the high-dose cohort (colored squares)

(A and B) Endurance. Endurance by 6 min walking test (6MWT; A) and 3 min stair climb test (3MSCT; B) were performed before (when subjects were under enzyme replacement therapy [ERT]) and after gene therapy administration. No statistically significant changes were observed in the median 6MWT ($p = 0.125$) and 3MSCT ($p = 0.625$) at last follow-up evaluations compared to baseline. For participant 008, only the data collected before ERT restart are reported in the graph.

(C and D) Pulmonary function. Pulmonary function was evaluated by spirometry as forced expiratory volume in 1 s (FEV_1; percentage predicted, C) and forced vital capacity (FVC; percentage predicted, D) before (when subjects were under ERT) and after gene therapy. No statistically significant changes were observed in the median FEV_1 ($p = 0.125$) and FVC ($p = 0.250$) at last follow-up evaluations compared to baseline. For participant 008, only the data collected before ERT restart are reported in the graph. Normal FEV_1 and FVC predicted values ranged between 70% and 100%.[15] Patient identification numbers are shown in the key.

from mild to moderate tricuspid valve regurgitation in 007) (Table 3). No relevant changes in the Childhood Health Assessment Questionnaire disability index (CHAQ-DI) scores were observed (Table S4).

DISCUSSION

We report a multi-year follow-up of patients with MPS VI who received an intravenous infusion of high dose (i.e., 6×10^{12} gc/kg) AAV2/8.TBG.hARSB. As previously reported,[14] participants who received the low and intermediate vector doses also had sustained increases of serum ARSB but of lesser magnitude. However, their urinary GAG concentrations rose to values detected in untreated MPS VI patients, thus mandating the reintroduction of ERT.[14] In the long-term follow-up study, with the exception of one patient who reintroduced ERT because of an increase in urinary GAG concentrations, patients in the high-dose cohort showed sustained urinary GAG concentrations below the threshold corresponding to the average concentrations of untreated patients from a historical cohort.[17] Only one patient in the high-dose cohort required reintroduction of ERT. It is notable that this patient achieved the highest expression of serum ARSB after gene therapy, but this gradually declined after week 15 with a concomitant increase in urinary GAGs. These changes occurred without variation in the anti-ARSB antibody titer. Moreover, the participant did not report any symptoms or show signs of worsening of her underlying disease. Overall, high-dose cohort participants showed serum ARSB values of 38%–67% of mean healthy values that were sustained over the observation period. Importantly, this long-term study confirmed that intravenous infusions of the AAV vector were not associated with an increase in adverse AAV vector-related events.

Since its approval by the US Food and Drug Administration in 2005 and the European Medicines Agency in 2006, several clinical trials and observational studies confirmed the long-term safety and clinical efficacy of ERT in terms of reductions in urinary GAGs and improved endurance and pulmonary function. Preclinical and clinical studies support urinary GAGs as a biomarker of MPS VI because their reduction correlates with clinical benefit after ERT or gene therapy.[7,18–21] Although consistent correlations have been observed between urinary GAG concentrations and GAG clearance in the kidney and other tissues or clinical outcomes, there is no clear threshold in urinary GAG concentrations that can predict meaningful changes in specific clinical endpoints. Nevertheless, urinary GAGs are widely used and continue to be the most reliable biomarker for MPS VI and other mucopolysaccharidoses.[22] Previous studies have shown that urinary GAG excretions >200 mg/mg creatinine are

Table 2. Liver and spleen sizes in study participants of the high-dose cohort

	Liver longitudinal diameter				Spleen longitudinal diameter			
	Baseline		Last follow-up[a]		Baseline		Last follow-up[a]	
Participant	mm	ct[b]	mm	ct[b]	mm	ct[b]	mm	ct[b]
01-006	110	60	113	60	87	80	77	45
01-007	118	75	120	65	70	15	74	20
01-008	111	55	122[c]	70[c]	89	85	101[c]	96[c]
01-009	102	45	109	55	65	7	79	60
Median[d]	110.5	57.5	113	60	78.5	47.5	77	45
Min[d]	102	45	109	55	65	7	74	20
Max[d]	118	75	120	65	89	85	79	60

Liver and spleen longitudinal diameters were measured by liver and spleen ultrasound before and after gene therapy administration. ct, centile.
[a]01-006, 01-007, and 01-008: 48 months; 01-009: 42 months.
[b]Centiles for liver and spleen size are based on previous studies.[16]
[c]Measurements performed after resuming ERT.
[d]Median, min, and max are calculated excluding measurements after ERT.

associated with an accelerated clinical course in untreated MPS VI individuals.[7] Our patients showed GAG excretions >200 mg/mg creatinine, but they have not manifested evidence of disease progression, questioning the relevance of this threshold as an indicator of worsening outcomes, at least in the context of gene therapy. Higher vector doses have been safely employed in other liver-directed AAV clinical trials,[23] and they have the potential to result in higher hepatocyte transduction efficiency and, hence, higher serum ARSB activities, enabling further reduction or normalization of urinary GAGs.

Untreated patients with MPS have impaired endurance and pulmonary function, whereas these parameters improved for up to 5 years in patients treated with ERT.[24] Therefore, clinical endpoints that included pulmonary function tests and endurance were met in this study. Pulmonary function showed slight but sustained improvements in the clinical trials for ERT,[25] but in long-term studies up to 3 years after starting ERT, these improvements were markedly attenuated.[24] In contrast to earlier clinical trials with ERT, having an untreated control group would not be ethically acceptable with either ERT or gene therapy. Therefore, the goal of this study was to investigate whether urinary GAG, liver/spleen sizes, endurance, cardiac, and lung functions remained stable after stopping ERT and administering gene therapy. Strikingly, the endurance tests showed no changes over the years after gene therapy. Instead, the outcomes of the lung function tests were more variable. Two participants (patients 006 and 009) showed no significant changes, whereas modest declines in FVC and FEV_1 were observed in the other two cases (patients 007 and 008), in whom FVC and FEV_1 decreased by 8%–11%, and 8%–13%, respectively. Endurance tests are readily reproducible, even in patients with slightly reduced cooperation,[26] whereas spirometry is effort dependent, with marked intra-patient variability.[15] Since poor effort can significantly affect FVC and FEV_1, we cannot be sure that even in the absence of overt airway disease, the compliance of patients 007 and 008 with the required breathing maneuvers was suboptimal. Longer follow-up is warranted to evaluate pulmonary function in these children with confidence. Cardiac function did not show significant changes in the study participants. However, a mild worsening of valvular disease was detected in two patients. This observation is consistent with previous findings in patients with MPS in whom valvular disease of the heart may progress despite ERT.[27]

In summary, a single administration of 6×10^{12} gc/kg of AAV2/8.TBG.hARSB, which is the highest dose of the trial, provided sustained hepatic expression of the therapeutic protein, consistent with the findings in individuals with hemophilia B.[23] Among the three participants who did not restart ERT, two were 10 years of age and one 5 years of age at the time they received the gene therapy. They were among the youngest participants in a liver-directed gene therapy clinical study, and yet they showed sustained ARSB serum levels and only a modest increase in urinary GAGs. Sustained transgene expression suggests that the rate of hepatocyte proliferation in these young patients does not cause significant dilution of the AAV episomal genome,[28] at least during the observation time of the present study. Furthermore, a single intravenous administration of AAV2/8.TBG.hARSB was not associated with adverse reactions for at least 3 years after gene therapy. Additionally, gene therapy was able to preserve endurance over a sustained period in the absence of ERT.

Limitations of the study

Limitations of study included the small study population and the absence of sex- and gender-based analyses. Although these limitations hamper the representativeness of the general population, limited sample size is a well-known issue with rare diseases.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Nicola Brunetti-Pierri (brunetti@tigem.it).

Materials availability

This study did not generate new unique reagents.

Table 3. Cardiac function and morphology in study participants enrolled in the high-dose cohort

	IVS (Z score)		LVEDD (Z score)		EF (%)		Aortic root dimension (Z score)		Tricuspid valve		Pulmonary valve		Mitral valve		Aortic valve	
	Baseline	Last fup[a]	Baseline	Last fup[a]	Baseline	Last fup[a]	Baseline	Last fup[a]	Baseline	Last fup[a]	Baseline	Last fup[a]	Baseline	Last fup[a]	Baseline	Last fup[a]
006	0.39	0.82	−1.14	−1.38	64	58	1.12	0.98	Mi R	Mi R	N	Mi R	Mi R	Mo R	Mo R	Mo R
007	0.26	−0.1	0.15	0.39	55	60	1.51	0.33	Mi R	Mo R	N	Mi R	Mi R	Mi R	N	N
008	0.84	1.2[b]	−0.35	–	55	63[b]	1.63	0.3[b]	Mi R	Mi R[b]	N	Na	Mi R	Mi Ra	Mo R	Na
009	0.61	0.49	2.2	1.99	65	61	2.15	2.56	Mi R	Mi R	N	N	Mo R	Mo R	Mo R	Mo R
Median[c]	0.5	0.49	−0.1	0.39	59.5	60	1.57	0.98	Mi R	Mi R	N	Mi R	Mi R	Mo R	Mo R	Mo R
Min[c]	0.26	−0.1	−1.14	−1.38	55	58	1.12	0.33	–	–	–	–	–	–	–	–
Max[c]	0.84	0.82	2.2	1.99	65	61	2.15	2.56	–	–	–	–	–	–	–	–

Cardiac data were collected before and after gene therapy administration. Aortic root dimension was assessed at aortic sinuses in telediastole. EF, left ventricle ejection fraction; IVS, interventricular septum; Last fup, last follow-up; LVEDD, left ventricular end-diastolic diameter; Mi, mild; Mo, moderate; N, normal; R, regurgitation.

[a]01-006, 01-007, and 01-008: 48 months; 01-009: 36 months.

[b]Measured after resuming ERT.

[c]Median, min, and max are calculated excluding measurements after ERT.

Data and code availability

Anonymized data that support this study are available for sharing and further examination from the lead contact upon reasonable request pending institutional review board approval. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request. This paper does not report original code.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.R., A.A., and N.B.-P.; methodology, A.R., S.Z., G.I.M., S.G., A.A., and N.B.-P.; validation, A.R., R.R., M.D'A., S.F., A.A., and N.B.-P.; formal analysis, A.R., R.R., M.G.V., S.G., A.A., and N.B.-P.; investigation, A.R., R.R., S.F., M.D'A., V.P., R.P., G.P., F.S., F.B., F.D., C.A.R., C.P., M.O., J.J.M., G.I.M., A.A., and N.B.-P.; data curation, A.R., R.R., M.D'A., V.P., R.P., S.Z., A.A., and N.B.-P.; writing – original draft, A.R. and N.B.-P.; writing – review & editing, R.R., S.F., M.D'A., V.P., R.P., S.Z., G.P., F.S., F.B., F.D., S.F., C.A.R., C.P., M.O., J.J.M., M.G.V., G.I.M., and S.G.; project administration, M.D'A., V.P., R.P., and S.Z.; funding acquisition, A.A. and N.B.-P. A.R., A.A., and N.B.-P. had unrestricted access to all data. All authors agreed to submit the manuscript, read and approved the final draft, and take full responsibility of its content, including the accuracy of the data and the fidelity of the trial to the registered protocol and its statistical analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHOD DETAILS
  - Safety data
  - Efficacy data
- QUANTIFICATION AND STATISTICAL ANALYSES
- ADDITIONAL RESOURCES

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-hARSB polyclonal antibody	Covalab, Villeurbanne, France	N/A
anti-hARSB antibody (sheep anti-hARSB polyclonal antibody)	R&D system	Cat# AF4415; RRID:AB_10730712
Chemicals and recombinant proteins		
1,9-dimethylmethylene blue chloride	Sigma Aldrich, St Louis, Missouri, USA	341088
rhARSB (Naglazyme),	BioMarin Pharmaceutical Inc.	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

This is a phase 1/2 open-label, dose-escalation trial investigating the safety and efficacy of AAV2/8.TBG.hARSB in patients with MPS VI who were at least 4 years of age (ClinicalTrials.gov number, NCT03173521; EudraCT number, 2016-002328-10). The gender, demographics and genotypes of these individuals are summarized in Table S1. The AAV2/8.TBG.hARSB vector is a recombinant AAV serotype 8 vector containing a single-stranded AAV2 genome (AAV2/8) encoding the human ARSB (*hARSB*) under the control of the thyroxine-binding globulin (TBG) promoter as described elsewhere.[14] Among the nine patients recruited, four received the high dose of $6x10^{12}$ gc/kg. The study was approved by regulatory authorities (the Agenzia Italiana del Farmaco for the primary center) and by the institutional review boards (246/16 University of Naples "Federico II" and 2017/09–46 KA-17037 Hacettepe University). Parents provided written informed consent, and the patients provided assent. The trial was conducted according to the Declaration of Helsinki and the principles of good clinical practice. Details on inclusion/exclusion criteria, study procedures and results for participants enrolled in the low and intermediate dose cohorts have been presented elsewhere.[14] This study was sponsored by the Telethon Foundation ETS and was designed by the last two authors; data were collected by the site investigators (listed as authors), who vouch for the fidelity of the trial to the study protocol. Efficacy and safety outcomes have been previously reported up to 28 months in the whole study sample. All participants in the high-dose cohort received a prophylactic course of corticosteroids consisting of a single intravenous administration of methylprednisolone at the dose of 1.5 mg/kg (maximum 100 mg) the day before gene therapy (Day −1) and oral prednisolone at the dose of 1 mg/kg/day (maximum 60 mg/day) from the day of gene therapy administration up to 4 weeks post-gene therapy followed by tapering of prednisolone dose up to week 9 post-gene therapy.

Long-term data from the five participants enrolled in the low- and intermediate-dose cohorts are not herein shown. Patients enrolled in the high dose cohort are shown in the present study and they were evaluated every 6 months up to 48 months (208 weeks) after vector infusion.

METHOD DETAILS

Safety data

The primary objective of this study was to investigate the safety of the AAV2/8.TBG.hARSB vector. Safety assessments included physical examination and vital signs, recording of adverse events (AEs), and laboratory testing, including immunological assays.[14] Abdominal ultrasounds were evaluated by qualified personnel at each site at least once a year to monitor participants for liver fibrosis and other potential hepatic disorders including tumors. Long-term assessments included liver enzymes (aspartate transaminase [AST] and alanine aminotransferase [ALT]), α-feto-protein (AFP), and routine blood and coagulation parameters that were performed as standard laboratory tests at the diagnostic laboratory of Federico II University Hospital.[14] Total serum anti-AAV8 antibody titers were measured as previously described.[29] Briefly, on day 1, 96-well plates were seeded with 2x104 2V6.11 cells/well for 24 h in presence of ponasterone A (Life Technologies, Carlsbad, USA). A recombinant AAV8 vector encoding luciferase under the control of the cytomegalovirus (CMV) promoter (AAV8-CMV-Luc) was diluted in serum-free Dulbecco Modified Eagle Medium (DMEM, Life Technologies, Carlsbad, USA) and incubated with semi-log serial dilutions (1:1 to 1:3160) of serum samples for 1 h at 37°C. Subsequently, serum-vector mixtures were added at a multiplicity of infection of $2x10^2$ genome copies/cell to cells incubated in DMEM with 10% Fetal Calf Serum (FCS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C and 5% CO2. Each dilution was performed in triplicate. After 24 h, cells were lysed with the Bright Glo system (Promega, Madison, USA) and luciferase activity was measured by a luminometer (ENSPIRETM, PerkinElmer, Waltham, USA). Transduction efficiency was measured as Relative Light Unit (RLU) per second. The neutralizing titer was reported as the highest serum dilution that inhibited AAV transduction by $\geq$ 50% compared with the control without serum (100% transduction).

Efficacy data

The secondary objective of this study was to investigate the efficacy of the AAV2/8.TBG.hARSB vector. Biochemical outcomes included urinary GAG concentration and serum ARSB. Urinary GAG were measured by a method based on spectrophotometric detection of metachromatic changes to the dye 1,9-dimethylmethyleneblue resulting from GAG binding.[14] GAG concentrations were normalized to urinary creatinine concentrations which were determined separately on the same urine sample. At each time-point, urinary GAG and creatinine levels were measured three times independently on urine samples collected at least 24-h apart. Each urine sample was divided in two aliquots, one for urinary GAG analysis and the other for determination of creatinine concentration. The three values of GAG concentration normalized to urinary creatinine were averaged to obtain the urinary GAG concentration at that specific time point. The threshold to restart ERT was set at > 321g GAG/mg creatinine based on the average urinary GAG value observed in a large cohort of MPS VI subjects without ERT.[17]

Active ARSB was measured in serum by an immune-capture assay based on the use of a specific custom-made anti-hARSB polyclonal antibody (Covalab, Villeurbanne, France), as previously described.[11] 96-well plates (Nunc ImmunoTm Micro-well, SigmaAldrich St. Louis, Missouri, USA) were coated with 5 μg/mL of anti-hARSB antibody in 0.1 M NaHCO3 and incubated overnight at 4°C. The following day, plates were blocked with 1% milk 0.25 M NaCl/0.02 M Tris pH 7.0; after 2 h of incubation at room temperature and a washing step, standard, samples and controls were added to each well. Plates were incubated at 4°C overnight. The following day, 5 mM 4-methylumbelliferylsulfate potassium salt (4-MUS; M-760-5, GoldBio, St Louis, Missouri, USA) substrate was added to each well and then incubated at 37°C for 4 h. Reactions were stopped by adding stop solution (0.2 M glycine pH 10.8). Plates were shaken for 10 min at room temperature and fluorescence was read (excitation of 365 nm/emission of 460 nm) on a multiplate fluorimeter (Infinite F200; TECAN, Männedorf, Switzerland). Serum active ARSB is expressed as picograms per milliliter (pg/mL) of serum, using a standard curve made of rhARSB (Naglazyme, BioMarin Inc, Novato CA, USA). Sera from five healthy subjects (3 females and two males) were used as normal controls. Percentage of active ARSB was calculated based on the mean of serum active ARSB measured in healthy subjects.

Total anti-hARSB antibodies were measured by a bridging electro-chemi-luminescent assay (Meso Scale Discovery, Gaithersburg, MD, USA), as previously described.[14] Briefly, samples were first serially diluted from 1:1 (no dilution) to 1:3,125 in the negative quality control (NQC), which is a pool of 7 healthy human sera not associated with a detectable signal and then further diluted 1:10 in the assay diluent. rhARSB (Naglazyme, BioMarin Pharmaceutical Inc.) was conjugated with either biotin or ruthenium using the Meso Scale Discovery (MSD) biotin and gold sulfo-tag (or ruthenium) conjugation packages as recommended by the supplier (Meso Scale Discovery, Rockville, MD, USA). Biotinylated and ruthenylated rhARSB were mixed 1:1 to a final concentration of 4 μg/mL/each. Samples or controls were mixed 1:1 with the biotinylated/ruthenylated rhARSB mix and incubated for 2 h at room temperature with shaking. In the meantime, streptavidin plates were blocked with blocking solution for 1 h at room temperature with shaking. Samples and controls (50 μL) were added in duplicate to streptavidin plates and incubated for 1 h at room temperature with shaking. After washing, a read buffer was added and chemiluminescence was read by a Quickplex instrument (Meso Scale Discovery). Each plate included the NQC, a high-quality control and a low-quality control in triplicate, prepared by diluting an anti-hARSB antibody (sheep anti-hARSB polyclonal antibody, AF4415, R&D system) in the NQC at the concentrations of 50 and 1 μg/mL, respectively. Titers were calculated as the reciprocal dilution crossing the cut point (NQC*1.12 luminance raw value). If the dilution 1:31,250 was still associated with a value above the cut point, the titer will be released as ≥ 31,250; if the loss of signal was <10% of the luminance raw value measured at 1:10 dilution, the titer was indicated as not quantifiable; if the 1:10 dilution value was < the cut point, the titer was reported as undetectable.

Clinical outcomes included: i) endurance as evaluated by the 6MWT and the 3MSCT; ii) pulmonary function as measured by forced vital capacity (FVC; % predicted) and forced expiratory volume in 1 s (FEV_1; % predicted); iii) cardiac morphology and function as evaluated by cardiac ultrasound with Z-scores [interventricular septum (IVS), left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), and aortic root dimension]; and iv) liver and spleen size as measured by abdominal ultrasound. Patient-related outcome measures included health-related quality of life as assessed by the Childhood Health Assessment Questionnaire [CHAQ-DI].

QUANTIFICATION AND STATISTICAL ANALYSES

The data presented were collected from study initiation to 31 January 2024. Outcomes were collected at specific time points, but when extra visits were performed, additional data were included in the analyses. Continuous variables were described with mean (±SD, Standard Deviation, or ±SE, Standard Error) or median (min-max), as appropriate, while qualitative variables with count or percentage. No formal sample size calculation was performed due to the nature of the study. Paired comparisons (i.e., baseline vs. last follow-up data) were performed by Wilcoxon Rank-Sum test (two-sided at 5% significance level). Quantifications are reported in the figure and table legends, and statistical analysis is reported in the main text of the results section.

ADDITIONAL RESOURCES

The Phase I/II clinical trial is registered at https://clinicaltrials.gov/study/NCT03173521?term=NCT03173521&rank=1 and the registration number is NCT03173521.