Safety and activity of an engineered, liver-tropic adeno-associated virus vector expressing a hyperactive Padua factor IX administered with prophylactic glucocorticoids in patients with haemophilia B: a single-centre, single-arm, phase 1, pilot trial

Feng Xue, Huiyuan Li*, Xia Wu*, Wei Liu*, Feixu Zhang, Dingyue Tang, Yunfei Chen, Wentian Wang, Ying Chi, Jing Zheng, Zengmin Du, Wei Jiang, Chen Zhong, Jun Wei, Ping Zhu, Rongfeng Fu, Xiaofan Liu, Lingling Chen, Xiaolei Pei, Junjiang Sun, Tao Cheng, Renchi Yang, Xiao Xiao, Lei Zhang*

Summary

Background A novel, engineered, liver-tropic adeno-associated virus vector expressing a hyperactive Padua factor IX (FIX) protein (BBM-H901) has been developed and is promising for haemophilia B gene therapy. We aimed to explore its safety and activity in increasing FIX concentrations and reducing bleeding frequency.

Methods We did a single-centre, single-arm, phase 1, pilot trial evaluating the safety and activity of a single intravenous infusion of BBM-H901 at the Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College (Tianjin, China). We enrolled adult patients with haemophilia B (aged >18 years) with baseline FIX coagulation activity (FIX:C) of less than 2 IU/dL, no FIX inhibitor, and low titre of neutralising antibodies (≤1:4) against vector capsid. Eligible participants were intravenously infused with a single dose of 5×10^{12} vector genomes (vg)/kg of BBM-H901 after 1 week of prophylactic prednisone treatment (1 mg/kg per day). Primary endpoints were the incidence of treatment-related adverse events, change in alanine aminotransferase (ALT) and aspartate amino transferase (AST), and development of antibodies against vector capsid within 1 year of infusion. We report the results of the prespecified 1-year analysis following complete enrolment. The trial is registered with ClinicalTrials.gov, NCT04135300, and is complete.

Findings Between Oct 16, 2019, and Jan 13, 2021, 12 male participants were assessed, and ten Chinese participants were enrolled and infused with BBM-H901. After a median follow-up of 58 weeks (IQR 51·5–99·5), mean FIX:C reached mean 36·9 IU/dL (SD 20·5). No serious adverse events, no grade 3–4 adverse events were observed. Grade 1–2 adverse events related to BBM-H901 include pyrexia (1 [10%]) and elevation of aminotransferase(1 [10%]). No FIX inhibitors were observed. All participants developed antibodies against vector capsid after infusion. Eight (80%) participants had ALT and AST concentrations below the upper limit of normal throughout the follow-up period. Two (20%) participants had elevation of ALT and AST accompanied with decrease of FIX:C, which remained at 7 IU/dL and 11.8 IU/dL, respectively.

Interpretation This pilot study suggests that liver-tropic BBM-H901 is safe 1 year after infusion. Vector derived FIX:C concentration is sufficiently high to prevent bleeding events and minimise the need for replacement therapy in small populations with haemophilia B. These findings support further study.

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Introduction

Haemophilia B is a monogenic inherited bleeding disorder caused by a mutation in the *F9* gene that results in lifelong excess bleeding, typically in joints and muscles, leading to crippling arthropathy.[1,2] Gene replacement therapy by means of adeno-associated virus (AAV) vectors has been long sought and numerous human clinical trials have been done. Two trials[3,4] of AAV-mediated FIX gene transfer to the liver have achieved sustained vector-derived FIX expression at therapeutic levels, whereas another[5] did not, possibly because of immune responses against AAV capsid or 5□-C-phosphate-G-3□ (CpG) motifs in the coding sequence.[3,4,5]

Cellular immunity against transduced hepatocytes, usually in a vector-dose-dependent manner, might cause asymptomatic transaminitis that is often associated with decline or even complete loss of FIX expression.[6,7] To increase success of haemophilia B gene therapy, use of

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*Contributed equally as joint first authors

State Key Laboratory of Experimental Hematology, National Clinical Research Center for Blood Diseases, Haihe Laboratory of Cell Ecosystem, Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin Key Laboratory of Gene Therapy for Blood Diseases, Chinese Academy of Medical Sciences Key Laboratory of Gene Therapy for Blood Diseases, Tianjin, China (F Xue MD, H Li MD, W Liu MD, Y Chen MD, W Wang PhD, Y Chi PhD, J Wei PhD, P Zhu PhD, R Fu MD, X Liu MD, L Chen BS, X Pei PhD, T Cheng MD, R Yang MD, L Zhang MD)**; East China University of Science and Technology, Xuhui District, Shanghai, China** (X Wu PhD, F Zhang PhD, D Tang PhD, Z Du PhD, W Jiang PhD, C Zhong PhD, J Sun PhD, X Xiao PhD)**; Belief BioMed, Xuhui District, Shanghai, China** (J Zheng PhD, X Xiao PhD)

Correspondence to: Dr Lei Zhang, State Key Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin 300020, China

zhanglei1@ihcams.ac.cn

or
Dr Xiao Xiao, East China University of Science and Technology, Xuhui District, Shanghai 200237, China
xiaoxiao@beliefbiomed.com
or
Dr Renchi Yang, Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin 300020, China
rcyang@ihcams.ac.cn

Research in context

Evidence before study

We searched PubMed for articles published in English reporting adeno-associated virus (AAV)-based gene therapy for haemophilia from database inception until Dec 12, 2021. We used the term "hemophilia", and "AAV", and "gene therapy", and "clinical trial". We also searched ClinicalTrials.gov for related past and ongoing clinical trials.

From the articles reporting results of clinical trial about gene therapy for hemophilia B, we found that AAV mediated liver-directed gene therapy provided sustained and stable endogenous FIX concentrations without apparent peaks and troughs, thus benefiting patients clinically. AAV serotypes with high liver tropism and low prevalence of neutralising antibodies are essential to the success of gene transfer and to enable treatment of more patients. Some patients might lose FVIII–FIX expression accompanied with asymptomatic elevation of liver enzyme despite the addition of immunosuppressive drugs (mainly glucocorticoids). In all clinical studies, glucocorticoids were initiated after vector infusion to treat or prevent cellular immunity, which was regarded as the main cause of loss of expression of transgene. However, the optimal timing to start immuno-suppressive drugs remains unclear. INF-γ ELISpot was frequently applied to investigate cellular immunity against transduced hepatocytes, but its sensitivity and the time required to test remain a limitation.

Added value of this study

To our knowledge, this the first trial of AAV-based liver-directed gene therapy in patients with haemophilia B done in China. The vector used in this study is named BBM-H901, consisted of a novel bioengineered capsid that has a lower prevalence rate of pre-existing antibodies among populations than many natural AAV serotypes, and included a liver specific expression cassette expressing CpG-motif-free FIX Padua. This is also the first trial, to our knowledge, to use prophylactic prednisone 7 days before vector infusion. In addition to ELISpot, in an exploratory analysis, we used single-cell RNA sequencing to monitor the immune response to AAV and the effect of glucocorticoids on immunity before and after AAV vector administration.

With these measures, we were able to show that BBM-H901 achieved quick, robust, and long-lasting gene expression on a single vector dose of 5×10^{12} vector genomes per kg. We also did not observe any severe adverse events throughout the study. We discovered that patients with less activity had a higher area under the curve for ALT and AST in the 26 weeks of treatment. Exploratory single-cell RNA sequencing suggested that glucocorticoids might suppress the immune response to AAV through several mechanisms.

Implications of all the available evidence

Our study support the safety and activity of the novel liver-tropic AAV vector. Prophylactic use of glucocorticoid before infusion can efficiently suppress the cellular immune response against AAV and ensure the activity of gene therapy. Single-cell RNA-seq could be used to assess the immune response against AAV capsid or other components over time after infusion.

liver-tropic AAV capsids for improved delivery efficiency and reduced immunogenicity have become desirable. Furthermore, a liver-specific expression cassette encoding a hyperactive FIX variant allowed for lower vector dosage and hence reduced vector-associated side-effects.[4] Glucocorticoid treatment after vector infusion is a common approach to inhibit AAV capsid-induced decline or loss of transgene expression; nonetheless this approach did not always work.[4,6,7] Whether prophylactic glucocorticoid use before vector infusion is effective has not yet been reported.

BBM-H901 is a novel vector comprised of an engineered liver-tropic AAV capsid (AAV843) and a synthesised liver-specific promoter and CpG reduced FIX Padua coding sequence packaged in an AAV particle as a double-stranded AAV genome. In a preclinical study (unpublished data), BBM-H901 was administered to *F9* knockout mice by tail vein. The FIX activity significantly increased to 97·4% ($5·0 \times 10^{11}$ vg/kg) and 338·96% ($1·0 \times 10^{12}$ vg/kg) at 1 week after infusion and persisted for the duration of the experiments (28 weeks). There was an apparent dose-efficacy correlation.

On the basis of preclinical studies, we launched a phase 1 trial on *F9* gene transfer using BBM-H901. In contrast with all previous studies in this setting,[2,4,7,8] a prophylactic glucocorticoid treatment 1 week before AAV vector infusion was explored to reduce the potential immune response against AAV capsid.

Methods

Study design and participants

This single centre, single-arm, phase 1, pilot trial evaluated the safety and activity of a single intravenous infusion of BBM-H901. We enrolled adult patients at the Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College (Tianjin, China) with haemophilia B (aged >18 years) with the following eligibility criteria: baseline FIX:C of less than 2 IU/dL, at least 100 exposure days to FIX concentrates, no history of FIX inhibitor, low titre of neutralising antibodies (≤1:4) against BBM-H901 capsid, and acceptable laboratory values (haemoglobin ≥11 g/dL, platelets ≥100 000 cells/μL, AST, ALT, and alkaline phosphatase no more than two times the upper limit of normal [ULN], bilirubin no more than three times the ULN, and creatinine ≤2·0 mg/dL). Because of vector shedding in semen, participants were eligible only if they were willing to use a reliable barrier contraception

method for 1 year after vector infusion. Patients on either prophylaxis or episodic treatment of FIX concentrates before enrolment were eligible.

Patients were excluded if they had hepatitis B or C virus infection, ALT and AST value greater than two times the ULN, underlying liver diseases (eg, previously diagnosed with portal hypertension, splenomegaly, encephalopathy, serum albumin reduction, or liver fibrosis stage ≥3), or had other chronic conditions that the investigators considered to constitute an unacceptable risk.

Assessment for inclusion and exclusion was based on patients' medical history, physical examination, complete blood counts, serum biochemistry, factor activity assay, Bethesda inhibitor assay, and hepatitis B and C tests. Patients who had participated in a gene therapy research trial within the past 52 weeks or in a clinical study with an investigational drug within the past 12 weeks were excluded.

This study was monitored by an independent safety monitoring committee comprised of three experts outside the clinical research centre. The safety committee was responsible for the evaluation of safety data and provided opinions on whether the study could be safely continued. The study protocol (appendix) was reviewed and approved by the Institutional Review Board of the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Science and Peking Union Medical College. This trial was done in full accordance with the Declaration of Helsinki. Written informed consent was provided by all participants before screening.

Procedures

The schematic diagram of study progression is shown in the appendix (p 9). All eligible participants received a single intravenous dose of 5×10^{12} vector genomes per kilogram bodyweight (vg/kg) of BBM-H901 over 1 h after 1 week of prophylactic daily oral prednisone at 1 mg/kg. The initial dose (1 mg/kg) lasted 2 weeks and tapered from day 8 after vector infusion. The total prednisone course was around 8–10 weeks. The use of prednisone could be resumed on the basis of liver function and FIX:C during follow-up. Patients were allowed to receive FIX concentrate replacement on demand or as prophylaxis as required after BBM-H901 infusion. For safety reasons, the second participant was dosed at an interval of 8 weeks after the safety of the first participant was confirmed; similarly, for the third participant. In our initial protocol, participants received one dose of 50 IU/kg recombinant factor IX (rFIX) infusion after 4 days washout on day 0 before vector infusion. We later cancelled the rFIX infusion on day 0, as approved by the institutional review board, on the basis of data from the first three participants that vector derived FIX:C rapidly increased to a high level at 1 week. Another protocol amendment, approved by the institutional review board, was the implementation of single-cell sequencing to monitor the effect of prednisone and BBM-H901 on the immune system.

After vector infusion, participants were followed up according to the schedule in the appendix (p 9). Briefly, participants had a hospital visit weekly from day 0 to week 8, every 2 weeks to week 18, and then in weeks 22, 26, 32, 38, 42, and 52. After 1 year of follow-up, participants entered the extension visit period. At each visit in the first year, we did a physical examination, evaluated adverse events and concomitant medication use, and collected blood samples for complete blood counts, serum biochemistry, and coagulation studies. Our reference range for ALT and AST is 0–50U/L, in accordance with Chinese Reference intervals for common clinical biochemistry tests and verified by the local laboratory. Adverse events were graded in accordance with the Common Terminology Criteria for Adverse Events, version 5.0. The schedule and contents of visit for follow-up visits are described in the protocol (appendix). A participant could be removed from the study for the following reasons: unresolvable grade 3–4 drug-related adverse events or other serious medical conditions unrelated to the study drug, non-compliance and unforeseeable conditions that substantially impede study data collection, or participant's willingness to withdraw from the study with informed consent.

See Online for appendix

The vector, BBM-H901, consists of a novel liver-tropic capsid (AAV843, also named XL32.1) isolated from a previous published AAV capsid library[9] (amino-acid sequence 98% homologous to AAV1 and AAV6) and a double-stranded AAV gene expression cassette (FIX-901) 2248 bp in length (from left to right inverted terminal repeat sequences). FIX-901 contains a 188 bp fully synthetic liver specific promoter, followed by a synthetic intron of 88 bp and a CpG motif-reduced mutated Padua (Arg338Leu) *F9* coding sequence containing an internal synthetic intron of 83 bp and by a 125 bp polyA signal sequence at the 3□ end of the cassette (appendix p 9).[4,7] The detailed information on gene shuffled AAV843 capsid origin and characteristics, protein sequence, full gene expression cassette sequence, and methods of production of BBM-H901 are available in the appendix (pp 2–4). In brief, the vector was produced in HEK293 cell suspension culture (200 L per batch) and the traditional triple-plasmid transient transfection method in serum-free chemically defined culture media. The viral particles were purified by a two-step chromatography method without ultra-centrifugation. Vector potency assays were done in vitro, using cell culture, and in vivo, using the *F9* gene knockout haemophilia B mouse model, by measuring FIX:C by one-stage aPTT method. Genome titres were established by qPCR using optimised PCR primers detecting the coding sequence of *F9* transgene. A more specific description of production and purification, vector DNA titre, and potency assays is available in the appendix (pp 2–3). The percentage of full particles was

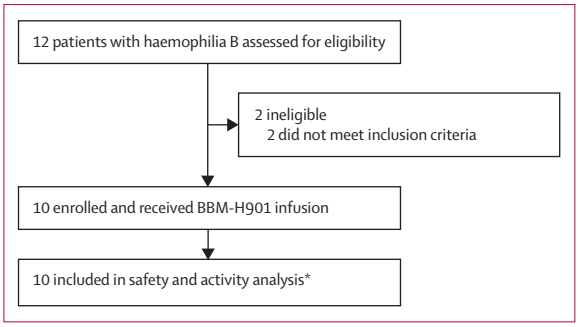

Figure 1: **Trial profile**
*Follow-up period for safety is 1 year of vector infusion. All participants are followed-up for vector derived FIX concentration now.

more than 80%, which was mainly established by analytical ultracentrifugation.

Single-cell RNA sequencing was done on blood samples taken from participants 4–10 according before prednisone, before vector infusion, 7 days after vector infusion, and 14 days after prednisone withdrawal (appendix p 9). The detailed descriptions of sample preparation, single-cell RNA sequencing, data preprocessing, quality control, T cells/antigen presenting cells (APCs) subset identification, Gene Set Enrichment Analysis (GSEA), Gene Set Variation Analysis (GSVA), and cell-chat analysis are available in the appendix (pp 5–7). The single-cell RNA sequencing data have been deposited in the NCBI Gene Expression Omnibus database under the accession code GSE190760.

AAV-capsid specific T cells were detected by IFN-γ ELISpot assay; a detailed description is provided in the appendix (pp 6–7). Capsid-specific T cell responses were considered positive if the number of spot-forming units (SFUs) per million cells was more than 50 and at least three-fold higher than the unstimulated negative control.

Outcomes

The primary endpoint was the safety of BBM-901, including incidence of treatment-related adverse events within 1 year after infusion, changes in ALT and AST, and development of antibodies against the AAV843 capsid. The secondary endpoint was the vector-derived FIX:C, measured by one-stage aPTT method (appendix p 2). Exploratory prespecified endpoints were residual vector genomes in body fluids (specifically, vector shedding in blood, saliva, urine, and semen, measured with qPCR), change in number of target joints, annualised bleeding rate (number of bleeding events per year), annualised number of FIX infusion 1 year before and after vector infusion, and immune status change after glucocorticoid treatment.

Statistical analysis

This pilot study was on haemophilia B, a rare disease with estimated prevalence of 1 in 25 000 male births. Therefore, a sample size calculation was not done. The investigators aimed for ten participants, mainly on the basis of common practice for sample sizes of phase 1 trials of gene therapy for rare diseases—eg, 3+3+3 patients in vector dose exploratory studies. We assumed that ten participants was a sufficient number to provide adequate safety and activity information for a pilot study.

Data from participants was analysed by means of descriptive statistics. Median (IQR) and mean (SD) were used to present numerical values. Clinical data collected after infusion (eg, annualised bleed rate, number of target joint, and number of FIX concentrate treatments) were compared with the data collected over the past 52 weeks before enrolment, by means of two-tailed paired *t* tests. In post-hoc exploratory analyses, an independent sample non-parametric test was used to compare the area under the curve (AUC) for ALT and AST between two groups arbitrarily defined on the basis of FIX:C. The two groups were the satisfactory activity group (FIX:C ≥40 IU/dL at 6 months) and inferior activity group (FIX:C <40 IU/dL at 6 months). A p value of less than 0·05 was considered statistically significant. All statistical analyses were done in GraphPad Prism 9.0. This study is registered with ClinicalTrials.gov, NCT04135300.

Role of the funding source

The funders of the study were not involved in study design, study conduct, data collection, data analysis, data interpretation, or writing of the report.

Results

Between Oct 16, 2019, and Jan 13, 2021, 12 male participants were screened and ten participants were enrolled into the study, with two patients excluded for having hepatitis C infection (figure 1). Participants' ages ranged from 20 years to 59 years (mean 29·5, SD 11·0; table 1). Before enrolment, six participants on prophylaxis had a mean annualised bleeding rate (ABR) of 8·3 (SD 3·5), and four patients on episodic treatment had a mean ABR of 41·3 (19·1). All ten participants were included in safety and activity analyses. The follow-up period for safety analyses was 1 year after vector infusion, and the median follow-up for activity analyses was 58 weeks (IQR 51·5–99·5).

Allergic and unwell reactions were not observed in any of the participants during the process of vector infusion. At the time of data analysis, participants were followed up for 705 weeks (range 50–117). No serious adverse event was reported. One adverse event deemed by the investigator to be probably related to the BBM-H901 was fever in one participant, which occurred 18 h after infusion (table 2). The fever resolved following the administration of glucocorticoids. Four participants had intermittent pain usually following vigorous exercise or a long walk, which was worse in the joints affected with chronic haemarthropathy. One participant had a trauma

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
Sex	Male	Male	Male	Male	Male	Male	Male	Male	Male	Male
Ethnicity	Chinese Han	Chinese Han	Chinese Han	Chinese Han	Chinese Han	Chinese Han	Chinese Han	Chinese Mongol	Chinese Han	Chinese Han
Height, cm	173	167	164	170	169	182	181	172	171	186
Weight, kg	72·9	91·2	73	58	76	73	74	85	64·5	77
Body-mass index	24·4	32·7	27·1	20·1	26·6	22	22·6	28·7	22·1	22·3
HBsAg	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
HCV antibody	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
HIV status	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
F9 mutation	c.1238G>A (p.Gly413Glu)	C.1183T>C (p.Phe385Leu)	c.484C>T (p.Arg162Ter)	c.114delC (p.Asn38LysfsTer4)	c.128G>A (p.Arg43Gln)	c.128G>A (p.Arg43Gln)	c.128G>A (p.Arg43Gln)	c.758G>A (p.Gly253Glu)	c.157delG (p.Glu53LysfsTer51)	c.679G>T (p.Val227Phe)
Baseline FIX:C, IU/dL	<1	1·6	<1	<1	0·8	0·8	0·8	1·3	0·7	0·4
Baseline FIX:Ag, ng/mL	104·8	41·4	134·5	130·12	2795·3	3615·4	3224·8	13·05	10·03	49·08
Cross-reacting material	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Negative	Negative	Negative
Binding antibody against AAV843 at baseline	1:200	1:50	1:50	1:50	1:400	1:50	1:50	1:50	1:50	1:50
Neutralising Antibody against AAV843 at baseline	1:2	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1
Replacement regimen	Prophylaxis; 10–20 U/kg, 1–2 per week	Prophylaxis; 15 U/kg, 1 per 1–2 weeks	Prophylaxis; 30 U/kg, 1 per week	Prophylaxis; 10 U/kg, 1 per week	Prophylaxis; 15–20 U/kg, 1 per 1–2 weeks	Prophylaxis; 15–20 U/kg, 1 per 1–2 weeks	Episodic treatment	Episodic treatment	Episodic treatment	Episodic treatment
Replacement therapy	PCC	PCC plus rFIX	PCC	PCC	PCC	PCC	PCC plus rFIX	PCC	PCC	PCC
Annualised bleeding rate, number per year	7	13	9	7	3	11	38	60	51	16
Number of FIX injections per year	90	54	40	62	37	53	38	77	73	16
Number of target joints	1	2	2	0	0	2	1	4	3	1

FIX:C=factor IX coagulation activity. FIX:Ag: factor IX antigen concentration. PCC=prothrombin complex concentrate. rFIX=recombinant factor IX. FIX=factor IX concentrates.

Table 1: **Baseline characteristics of individual patients**

to the head at week 19, which required sutures without FIX infusion, and there was no excess bleeding.

One bleeding episode was reported by one participant and was identified as aggravation of a previous haematoma. He received FIX concentrate ten times. The event and treatment plan for this patient is described in the appendix (p 8).

Eight participants had ALT and AST concentrations below the ULN throughout the follow up period. Two participants had ALT and AST elevation. One participant (participant 4) had ALT of 64·5U/L and normal AST at screening and asymptomatic elevation of ALT to 137·9 U/L and AST to 56·8 U/L before vector infusion 7 days after starting prednisone, which resolved 1 week after the prednisone dose was increased and bicyclol and polyene phosphatidylcholine administered. He had two more episodes of asymptomatic ALT elevation during follow-up (appendix p 7–8). At week 22, his ALT was at 2·24 times his own baseline but still within normal limit. Then the second course of prednisone was initiated to control the elevation of ALT and prevent the decline of FIX:C (described later). His ALT rose to 80·4 U/L at week 24 after 2 weeks administration of prednisone. After concomitant use of bicyclol and polyene phosphatidylcholine, ALT gradually returned to normal range.

At week 43, ALT rising to 250 U/L, and AST rising to 79 U/L after 1 week's administration of prednisone prescribed with the aim of preventing decline in FIX:C level (described later). The elevation of ALT and AST was resolved after concomitant use of glutathione and bicyclol. The other participant (participant 7) had ALT of 59 U/L 2 weeks after prednisone was tapered off (at week 10 visit) and reached 71·8U/L at week 12. He was prescribed a second course prednisone starting at 50 mg daily and tapered over 5 weeks, and his ALT reduced to

	Number of patients	Number of events
Adverse event related to BBM-H901†		
Pyrexia	1 (10%)	1
Elevation of aminotransferase	1 (10%)	1
Adverse event related to prednisone†		
Acid reflux	2 (20%)	2
Thirsty	2 (20%)	2
Insomnia	2 (20%)	2
Acne	1 (10%)	1
Swallowing discomfort	1 (10%)	1
Nocturia	1 (10%)	1
Flatulence	1 (10%)	1
Elevation of aminotransferase	1 (10%)	3
Adverse event related to haemophilia		
Arthralgia	4 (40%)	4
Iliopsoas haematoma	1 (10%)	1
Right thigh haematoma	1 (10%)	1
Adverse event unrelated to treatment		
Amygdalitis	1 (10%)	1
Diarrhoea	1 (10%)	1
Otitis externa	1 (10%)	1
Oral mucosal ulcer	1 (10%)	1
Pharyngitis	1 (10%)	1
Periodontitis	1 (10%)	1
Common cold	1 (10%)	1
Trauma on head	1 (10%)	1
Trauma on right knee	1 (10%)	1

Data are n (%) and n. *Period for observation of adverse events was from date of obtaining signed informed consent form to 1 year after BBM-H901 infusion. †The relationship between adverse events and BBM-H901 or prednisone was established by clinical investigators. There were no grade 3–4 adverse events, and no death during the whole study period.

Table 2: **Adverse events (grade 1–2; n=10)***

32·2U/L at week 14. Thereafter, there was no further increase in ALT or AST.

Vector DNA was rapidly cleared in the serum by at least 3 logs within 24 h after intravenous injection (figure 2C). The timepoints (days post vector dosing) at which vector DNA was undetectable in various body fluid specimens are shown in the appendix (p 19). Briefly, the timepoints for negative vector DNA detection were day 28 for plasma and saliva, and day 56 for semen. There was no detectable vector DNA in urine specimens at day 0 or at the subsequent visit.

Before BBM-901 infusion, none of the participants had neutralising antibodies against AAV843 capsid at greater than 1:4 dilution ratio, whereas two had pre-existing AAV capsid-binding IgG at 1:200 and 1:400. 1 week after infusion, all participants developed high titre binding IgG and neutralising antibodies against the AAV843 capsid that lasted for the entire follow-up period (appendix p 20). All participants remained negative for inhibitors to FIX at all timepoints.

1 week after BBM-H901 infusion, vector derived FIX:C quickly reached a mean of 57·1 IU/dL (SD 20·2), close to the peak FIX:C (mean 64·1 IU/dL, SD 22·5) at a median of 5 weeks (IQR 1–6) after infusion. FIX expression became detectable as early as 1 day after infusion (figure 2B; participants 4–10 did not receive any rFIX infusion before receiving vector, so that there was no exogenous FIX:C interfering with the detection).

At data cutoff, FIX:C reached a mean of 36·9 IU/dL (SD 20·5) followed for a median of 58 weeks (IQR 51·5–99·5; figure 2A). The first three participants recruited had mean FIX:C of 33·6 IU/dL (SD 3·51; figure 2A). Mean FIX:C of all participants excluding the two who had ALT elevation above normal range is shown in the appendix (p 9). Another five participants had stable FIX:C up to the most recent follow-up visits, without apparent fluctuation. FIX:C fluctuated on both patients with ALT or AST elevation. The first's FIX:C reached 42·1 IU/dL at week 6, but declined after week 10 and was 15·7 IU/dL at week 22. The addition of a second course of prednisone made FIX:C rise to 21·8 IU/dL at week 26 but dropped to 2·7 IU/dL at week 42. Then the third course of prednisone was initiated and FIX:C gradually rose to 30 IU/dL at week 50. At week 66, after initiation of the fourth course prednisone, his FIX:C was 7 IU/dL (appendix p 8). The second participant with ALT or AST elevation had a FIX:C of 76·7 IU/dL at week 3, which remained at 61·6 IU/dL at week 10, whereas his ALT increased from 10·1 U/L (week 8) to 59 U/L (week 10). At week 12, his ALT further increased to 71·8 U/L and his FIX:C fell to 35·2 IU/dL. After the second course of prednisone, his FIX:C further decreased but remained stable at 11·8 IU/dL between weeks 42 and 54.

The median number of target joints for all participants before vector infusion was 1·5 (IQR 0·8–2·3), which fell to 0 after infusion (p=0·0031; figure 2D). The median ABR before enrolment was 12 (IQR 7–41) and 0 after vector infusion (p=0·0092; figure 2E). The median number of FIX concentrate infusions before gene therapy was 53·5 (IQR 38–74) and 0 after (p<0·0001; figure 2F).

IFN-γ ELISpot analysis of participants with ALT and AST elevation were negative at all timepoints for the first. The second, however, had 72 SFU per million cells at the same time as his decline in FIX:C. In one participant without ALT or AST elevation, the IFN-γ ELISpot was found positive at 7 and 8 weeks (76 SFU per million cells and 72 SFU per million cells) after vector infusion, but there was no elevation of ALT nor decline of FIX:C. All four other participants with constant and stable FIX:C level show negative results at all timepoints.

Correlation analysis showed no statistical association between ALT and AST and FIX activity (ALT r=–0·189, p=0·051; AST r=–0·108, p=0·27). We further compared the AUCs of ALT and AST in 26 weeks of treatment between the inferior activity group (n=3) and satisfactory activity group (n=4) by independent sample non-parametric test. The AUC of ALT was 432·83 (107·97) in

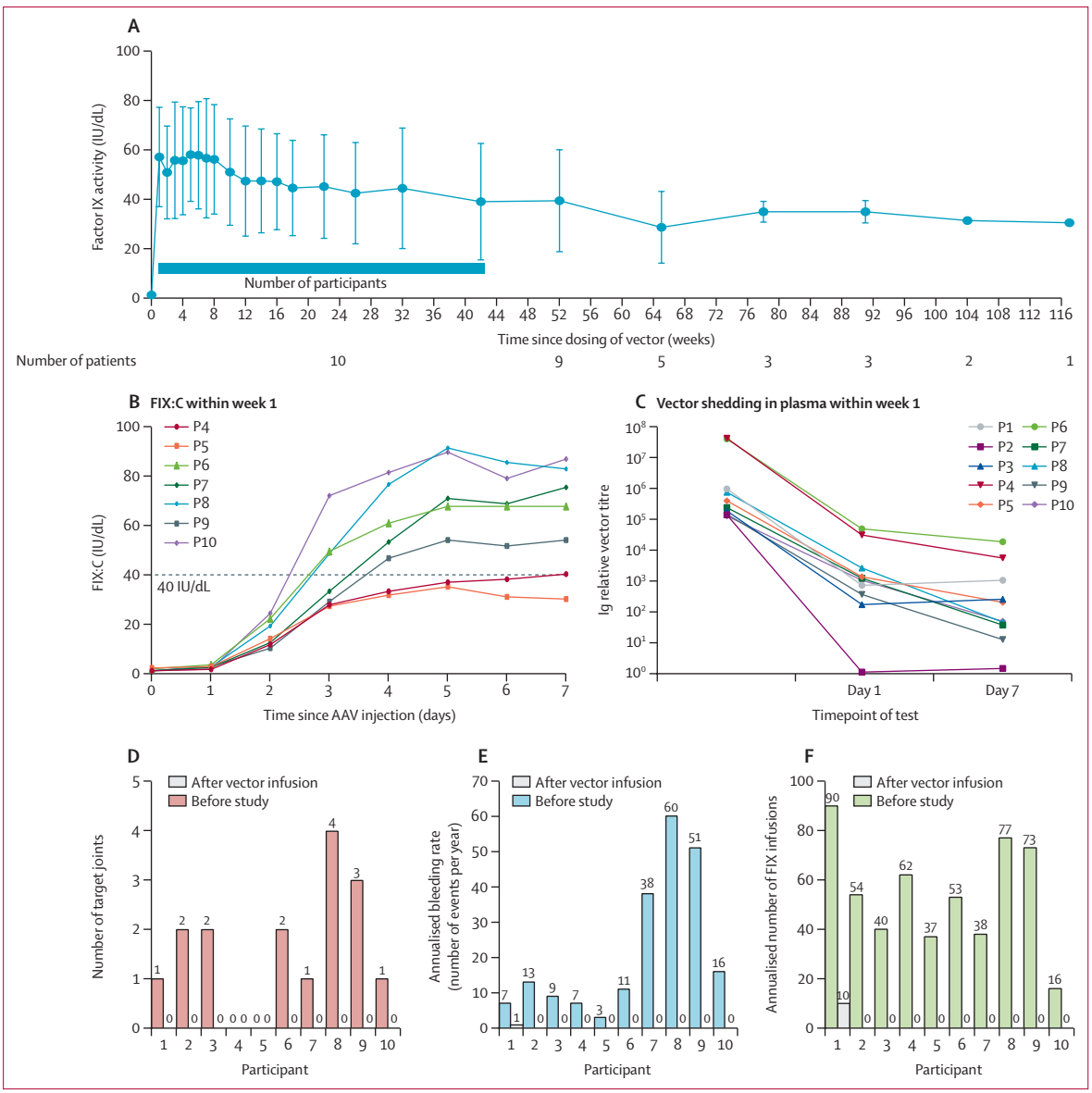

Figure 2: **Factor activity, vector shedding in plasma, and clinical parameters before and after vector administration**
(A) Vector derived factor IX activity (mean [SD]) over time. Number of participants at each timepoint is shown above the x-axis. (B) Vector derived FIX:C in the first week after gene therapy. The horizontal red dot line represents 40 IU/dL, which is the threshold for diagnosis of haemophilia B. (C) Vector shedding in plasma within 1 week. (D) Number of target joints before enrolment and after gene transfer. (E) Annualised bleed rate before enrolment and after gene transfer. (F) Infusion times of factor IX concentrate before enrolment and after gene transfer. FIX:C=FIX coagulation activity.

the inferior activity group and 254·15 (38·23) in the satisfactory activity group, p=0·025. AUC of AST was 305·67 (45·31) in the inferior activity group and 245·3 (13·15) in the satisfactory activity group, p=0·048 (appendix p 10).

By use of single-cell RNA sequencing (appendix pp 10–11), we identified ten subclusters of T cells based on their signature genes. Gene ontology analysis of differentially expressed genes of cytotoxic cells in the satisfactory activity and inferior activity groups showed that the top 20 enriched processes were mainly associated with immune responses (appendix pp 11–12). Thus, we further compared the immunology status of cytotoxic T cells between patients in the satisfactory activity and inferior activity groups and found that gene set scoring of cytotoxic T cell response to IFNγ–IL-6–IL-7 regulation modules were increased in the inferior activity group (appendix pp 13–14).

The differences in functional signatures of $CD4^+$ T cells, $CD8^+$ T cells, and antigen presenting cells (APCs) between the two groups were also analysed. Gene sets involved in T cell activation, proliferation, differentiation, and inflammatory factor secretion were significantly enriched on day 7 in each $CD4^+$ T cell and

CD8+ T cell subpopulation in patients in the inferior activity group (appendix pp 13–14). Additionally, dendritic cell differentiation, antigen presentation ability, and type I interferon production gene sets were enriched in each APC subset of patients in the inferior activity group on day 0 (appendix pp 13–14).

Glucocorticoids substantially reduced the proportion of cytotoxic T cells, while having no effect on percentage of CD8+ T cells in CD3+ T cells by single cell RNA-sequencing (appendix pp 13–14). Meanwhile, GSEA showed that transcripts associated with T cell activation, proliferation, response to IFNI, response to TNFα, and T helper 1 cell differentiation were significantly enriched at day –7 compared with day 0, and at stop GCs compared with day 7 (appendix pp 13–14). These results were confirmed with GSVA (appendix p 15), which also showed that the GSVA score of regulatory T cells (Treg) activation was elevated at day 7 compared with day –7. In addition, Treg subset identification based on single-cell RNA sequencing achieved the same results (appendix pp 13, 15). It is noteworthy that the GSVA score of T-cell mediated cytotoxicity in natural killer, γδT, and mucosal associated invariant T cells were elevated after AAV infusion (appendix p 15).

Dendritic cells, CD16+ monocytes, and classic monocytes were identified among APCs based on their signature genes by single-cell RNA sequencing. (appendix p 16). Glucocorticoid treatment substantially downregulated the percentages of CD16+ monocytes and dendritic cells (appendix p 17). Sequential GSEA showed that transcripts associated with APC differentiation, response to inflammatory factors, and antigen presentation ability were significantly enriched at day –7 compared with day 0, and at stop glucocorticoids compared with day 7 (appendix p 17). The GSVA produced similar results (appendix p 17).

Analysis using the CellChat R package suggested that the total secretion signal and cell–cell contact communication probabilities between CD16+ monocytes and T cell subsets were decreased during glucocorticoid administration (appendix p 17). Among them, the communication probabilities of CD86 cell–cell contact and soluble CD40 signalling between CD16+ monocyte and each CD4 or CD8 T subset were decreased at day 0 and day 7 compared with at day –7 or when patients stopped glucocorticoids. In addition, MHCII signalling between each APCs and CD4 subset showed a similar result.

Discussion

In this phase 1 study, we evaluated BBM-901, an AAV-based gene therapy vector, for its tolerability, safety, and activity in ten patients with haemophilia B from China. Throughout the study, no grade 3–4 adverse events were observed, and adverse events related to BBM-H901 (grade 2 pyrexia, an elevation of ALT and AST) only occurred in two participants and were resolved after short interventions. Similar to previously published studies, all participants developed high titre binding antibodies and neutralising antibodies against vector capsid. This implies that each participant only has one chance of receiving AAV-based gene therapy, unless a novel AAV capsid with no cross-reaction to serotypes that are currently being used or other interventions become available in the future. We also show no inhibitor against FIX Padua in our Chinese patients with haemophilia B, which is also a desirable safety feature.

Although many previous liver-directed gene therapies have shown detectable vector derived FIX:C and reduction in ABR and need for FIX concentrates, a major challenge for safety and activity was T cell immunity against AAV capsid proteins in the transduced hepatocytes or CpG oligodeoxynucleotides. The cassette used in this study was optimised with CpG oligodeoxynucleotides ablation, thus avoiding immunity against CpG as Konkle and colleagues proposed.[7]

Asymptomatic transaminitis often triggers the decline or even complete loss of vector derived FIX expression level. Some participants retained transgene expression when treated with glucocorticoids and others did not.[4,5,7] All previous studies used glucocorticoids after vector infusion either as prophylaxis or a treatment in response to transaminase elevation.[4,6,7,8] We instead initiated prednisone prophylaxis 7 days before AAV injection as a pre-emptive preventive measure. Glucocorticoids were tapered off in around 8 weeks. We reasoned that in addition to the benefit reducing T cell immunity before AAV vector injection, a pre-emptive glucocorticoid treatment could potentially protect participants from delayed discovery of liver enzyme elevation induced by cellular immunity against transduced hepatocytes after gene therapy. If a participant missed regular visits for whatever reasons (eg, travel restriction), they might be at risk of asymptomatic elevation of ALT and AST without knowing it and thus delay the initiation of glucocorticoid treatment. This is especially true in the developing countries and for residents in rural areas. We did observe some adverse events related to glucocorticoid prophylaxis, but all were mild at grade 1 or 2. Thus the potential benefit of this short-term glucocorticoid prophylaxis before vector infusion deserves further exploration. This approach should be studied further in a controlled study to establish the most optimised regimen.

Although in accordance with the national standard and verified in the local laboratory, our upper limit of normal for ALT and AST of 50 U/L is different from those in other countries (eg, 43 U/L, or more than 50 U/L).[4,8] Some studies also defined 1·5–2 times elevation from the patient's own baseline ALT value as indication for glucocorticoid treatment.[5] In our study, all the patients with ALT or AST concentration below 50 U/L had stable FIX expression, confirming that the ULN used in this study is appropriate. Regarding patients with ALT or AST elevation, we believe

the asymptomatic elevation of ALT above ULN in one patient during week 10 hospital visit was caused by cellular immune responses towards the AAV capsid. This notion is supported by the positive results of the IFN-γ ELISpot assay. The first had asymptomatic transaminase elevation several times, typically following administration of prednisone, suggesting underlying poor liver function. We speculated that prednisone seemed to elevate ALT and AST while somehow increasing the expression of transgene as well, suggesting that the transduced hepatocytes were not eradicated by cellular immunity in this participant. Research has shown that dexamethasone transiently enhanced transgene expression of transthyretin promoter in the mouse liver but not in dog liver, when glucocorticoid was administered following long-term AAV transduction.[10] Nonetheless, the BBM-H901 cassette did not contain any steroid response element, and FIX expression was not induced by glucocorticoid (unpublished data). Extended long-term observation is needed to investigate the outcomes of these participants.

Compared with other gene therapy trials for haemophilia B,[2,4,7,11] faster expression of transgene was observed in this study. We observed that hepatocytes transduced with BBM-H901 could express the transgene as early as 1 day after vector administration and rapidly approached peak FIX:C concentration within 1 week. This was true in seven patients that we monitored in this study. We believe that the rapid expression might be attributed to rapid delivery of gene into liver by the strong liver tropism of AAV843 capsid, because vector DNA in plasma was found to be rapidly cleared by at least three logs within 24 h, indicating the rapid liver uptake and rapid uncoating of the AAV843 capsid in addition to the double-stranded AAV ge nome vector genome structure. Rapid uncoating might favour rapid degradation of the capsid protein within the cells, and possibly reduce the delayed T cell immune response against capsid protein-containing hepatocytes.

In our study, we used a vector dose of 5×10^{12} vg/kg. This dose is four times lower than that used in the Uniqure study,[11] which used 2×10^{13} vg/kg. In the study by George and colleagues,[4] a relatively low vector dose of 5×10^{11} vg/kg was used. However, additional empty AAV particles in excess of 4–6 times as high as the full particles were added to the vector preparation to enhance the infectivity.[4] In terms of FIX:C, our study has achieved higher level expression at 1 year post-AAV infusion, because seven patients had FIX:C of greater than 30 IU/dL, and four patients had FIX:C of 50 IU/dL or greater. The high and steady vector-derived FIX:C obviously benefited all participants by preventing haemorrhage and ameliorating target joints without the need for injection of exogeneous FIX products.

We also found that the elevation of AST and ALT negatively correlated with FIX:C, suggesting that stronger cellular immunity might be the reasons for the unsatisfactory activity of gene therapy. Subsequently we examined AAV capsid-associated cytotoxic T-lymphocyte effect by IFNγ ELISpot assay. However, owing to the low frequency of AAV-specific T cells and the low sensitivity of the ELISpot assay,[12,13] we only found AAV-associated cytotoxic T-lymphocyte effect in participants 7 and 9. Thus, we used single-cell RNA sequencing to monitor AAV-specific immunity.

Through the sequencing, we found that functional signatures of each T-cell subset, especially the cytotoxic T cells at day 7 and APCs at day 0, were higher in patients in the inferior activity group, indicating that capsid-induced cytotoxic T lymphocytes could be the main contributory factor for reduced activity. Thus, to achieve long-term ideal expression of transgene, corticosteroid was given prophylactically from 7 days before vector infusion in this study. Consistent with reports by others, glucocorticoids dampened T-cell immune responses,[14–16] promoted Treg activation, suppressed antigen presentation ability of APC as well as the cell communication from APCs to T cells, effecting inhibition of capsid-specific cytotoxic T-lymphocytes. It is worth noting that, in this study, we found that ALT and AST changes in patients in the inferior activity group mainly occurred at 8–10 weeks (glucocorticoids had been discontinued at that time), accompanied by a decrease in FIX activity. But RNA sequencing results showed that the intensity of cellular immune responses in these patients on day 7 was already significantly higher than that in satisfactory activity group. Therefore, RNA sequencing is more sensitive than ALT and AST as an indicator. However, because of high cost, delayed results, which were not easy to obtain, and no unified baseline, the single-cell sequencing could not provide real-time advice for clinical management. In our study, single cell sequencing mainly helped us to uncover the specific mechanism of how glucocorticoid pre-treatment suppressed cellular immunity and to improve the activity. To sum up, our exploratory single-cell sequencing research suggested that glucocorticoid pretreatment effectively weakened T cells and APC functions before or after AAV injection. This could be the reason thus far that no complete loss of transgene expression in any of the ten patients was seen. These findings also add more justification for glucocorticoid treatment in advance of vector infusion.

Although this study provides data on the safety tolerability and activity of BBM-H901, the time period of this study is relatively short, thus the long-term clinical benefit (eg, improvement of arthropathy or pseudo-tumour) remains to be monitored for years to come, as the improvement of the above mentioned clinical parameter cannot be clearly observed within such a relatively short period. Extended observation will provide additional useful information (eg how long transgene expression will last, and if the joint health score improves, etc.). Additionally, the advantage of prophylactic use of glucocorticoids was not sufficiently shown for a small population of participants. Other limitations of this study

include the limited ethnic group and involvement of only a single centre. Further studies, enrolling more patients, are necessary to address the limitations of this study.

In summary, our results suggest that BBM-H901 is safe and active in patients with haemophilia B. Our exploratory single-cell RNA sequencing analyses also suggest that prophylactic glucocorticoid treatment reduced the baseline amounts of APCs, $CD4^+$ T cells and $CD8^+$ T cells, and upregulated Treg activation in patients with haemophilia B undergoing gene therapy. Therefore, this regimen of prophylactic administration of glucocorticoid could be useful in blunting the immune responses in other gene therapies in haemophilia patients.

Contributors

LZ, XX, RY conceived and designed the clinical trial. FX and WL executed the research, collected and interpret data, drafted the original manuscript. HL contributed to the RNA sequencing, data analysis, and writing and editing of the manuscript. XW contributed to the vector production, data analysis and editing the manuscript. YC, RF, XL, and LC contributed to patient follow- up and data collection. FZ and CZ contributed to the test of FIX antigen, binging antibody and neutralising antibody against AAV. DT and JS contributed to the detection of vector shedding. ZD, WJ, and JZ contributed to the vector production quality control. TC conceived the study and edited the manuscript. XP, WJ, PZ, YC, WW contributed to data analysis and editing of the manuscript. FX, HL, XW, WL, LC, XX, RY, and LZ accessed and verified the data. All authors had full access to raw data and had final responsibility for the decision to submit for publication. All authors reviewed, and approved the final version of this manuscript.

Declaration of interests

We declare no competing interests.

Data sharing

Qualified researchers can request the dataset, including de-identified individual participant data. Data can be requested from the corresponding authors from 12 months to 36 months after study completion. The study protocol is available as the appendix of this paper. The single cell RNA sequencing data have been deposited in the in the NCBI Gene Expression Omnibus database under the accession code GSE190760.

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