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ORIGINAL ARTICLE

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A phase 1/2 safety and efficacy study of TAK-754 gene therapy: The challenge of achieving durable factor VIII expression in haemophilia A clinical trials

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Abstract

Introduction: Haemophilia A is an X-linked bleeding disorder resulting from a deficiency of factor VIII (FVIII). To date, multiple gene therapies have entered clinical trials with the goal of providing durable haemostatic protection from a single dose. TAK 754 (BAX 888) is an investigational AAV8-based gene therapy containing a FVIII transgene. Reduction in CpG motifs was performed to reduce immunogenicity based on prior observations. Here, we describe the results of the first two cohorts treated with TAK 754.

Aim: To report clinical and translational results of the TAK-754 phase 1/2 AAV gene therapy study for the treatment of haemophilia A.

Methods: A phase 1/2 single arm open-label dose escalation study of TAK-754 was performed in participants with severe haemophilia A (NCT03370172). Participants

were monitored for safety events, endogenous FVIII activity and bleeding rates. Glucocorticoids were implemented to preserve transgene expression. A transcriptomics analysis was performed to evaluate immunogenicity along with additional post-hoc analyses.

Results: Four participants were dosed in two cohorts. Infusion of TAK 754 was well-tolerated. All participants developed mild transient transaminase elevation and subsequent loss of FVIII expression within the first 12 months of treatment despite use of glucocorticoids. Transcriptomic analysis did not demonstrate significant changes in immunogenicity signals in peripheral blood. One serious adverse event of hypophosphatemia occurred in the second cohort without obvious risk factors.

Conclusions: Sustained FVIII expression remains a challenge in haemophilia A AAV gene therapy trials. Mechanisms of transgene expression loss require further study as clinical studies enter long term follow-up periods.

KEYWORDS

adeno-associated virus, gene therapy, haemophilia, hypophosphatemia, phase 1–2

1 | INTRODUCTION

Haemophilia A is an X-linked bleeding disorder characterized by a genetic deficiency of coagulation factor VIII (FVIII). Severe haemophilia A patients require lifelong treatment with prophylactic FVIII replacement or other haemostatic therapies to prevent serious bleeding in joints and other tissues.[1–3] With the development of gene therapy technology based on the adeno-associated viral vectors (AAVs) for gene transfer, sustained FVIII expression and bleed prevention has been achieved after a single dose.[4,5] As the clinical experience with gene therapy has evolved, different patterns of FVIII activity have been observed. Some patients have achieved a mild haemophilia phenotype that provides protection from most spontaneous bleeding but does not fully normalize FVIII expression. While some gene therapies have achieved high initial FVIII expression followed by significant loss of expression that have required resumption of prophylaxis,[5,6] others have demonstrated a lower initial peak with more persistent long-term expression.[7–9] Challenges in achieving stable and durable multi-year FVIII transgene expression have been attributed to differences in capsid potency, transgene immunogenicity, the unfolded protein response, and promoter sensitivity.[10]

TAK-754 (BAX 888) was developed as a gene therapy for haemophilia A based on an AAV 2/8 capsid with a B-domain deleted codon optimized factor VIII transgene (FVIII-SQ) produced in HEK293 cells under the control of a liver specific promoter. In addition, CpG reduction of the transgene cassette was performed to avoid triggering the innate immune response that has previously been associated with loss of expression in a haemophilia B gene therapy with the same capsid.[11] Here, we present the results of four participants with haemophilia A treated with TAK-754 in a first-in-human study (NCT03370172).

2 | METHODS

TAK-754 consists of a recombinant adenovirus-associated virus 2/8 (AAV2/8) produced in HEK293 cells to deliver a single stranded codon-optimized B-domain-deleted (BDD) factor VIII (FVIII; FVIII-SQ[12]) transgene with a liver-specific transthyretin (TTR) promoter with flanking AAV2-derived inverted terminal repeats (Figure 1A).

2.1 | Study population

Male participants ages 18–75 years with severe haemophilia A (FVIII activity < 1%) were eligible for enrolment if they had an annualized bleeding rate (ABR) of ≥3 or were using FVIII prophylaxis and had at least 150 exposure days to FVIII concentrates. Key exclusion criteria were a history of inhibitors to FVIII (defined as a titer of ≥.6 Bethesda units) and the presence of AAV8 neutralizing antibodies based on a titer cutoff of ≥1:5. Participants could not have a known hypersensitivity to glucocorticoids since prednisolone was planned as an immunosuppression treatment. Participants were excluded if there was evidence of hepatic inflammation defined by ALT or AST > 1x upper limit of normal (ULN) of the reference lab, total bilirubin > 1.5xULN and direct bilirubin > .5 mg/dl, fibrosis Metavir stage F2 or greater based on liver biopsy or FibroSURE testing with a serum albumin below the lower limit of normal (LLN) and alkaline phosphatase > 2.0 x ULN. Participants were also screened for HIV, hepatitis B, hepatitis C, and autoimmune hepatitis.

2.2 | Study conduct

This study planned for up to three dose escalation cohorts in an open-label single arm design. Dosing was performed in the outpatient setting

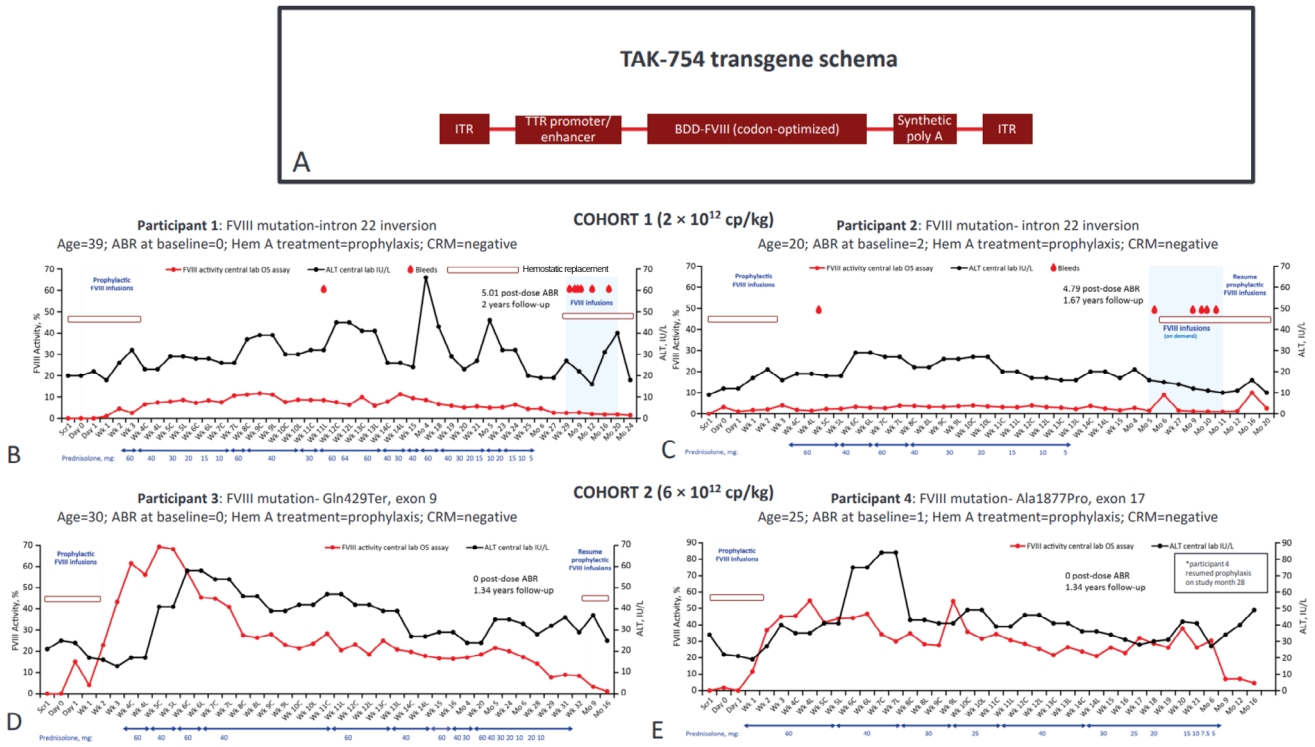


FIGURE 1 (A–E) TAK 754 vector design and individual participant study time course of factor VIII expression, ALT, bleeding, and immunosuppression. TAK-754 vector design and individual participant time courses. A, TAK-754 transgene and expression cassette elements. B and C, Baseline characteristics and time course of study participants in cohort 1. D-E: Baseline characteristics and time course of study participants in cohort 2. Annualized bleeding rates are calculated based the number of reported bleeding events divided by the number of months in the reporting period and multiplied by 12. Per protocol, bleeding events include any spontaneous or traumatic bleed experienced during the interval. Participant 4 resumed prophylaxis with emicizumab at approximately +28 months of follow-up. ITR, inverted terminal repeat; BDD-FVIII, B-domain deleted factor VIII; TTR, transthyretin; AAV, adeno-associated virus; ABR, annualized bleeding rate; ALT, alanine aminotransferase; cp, capsid particles; CRM, cross-reacting material; C, central; L, local; FVIII, factor VIII; L, laboratory; OS, one-stage; SCR1, screening 1; Wk, study week.

with sentinel dosing and a minimum of 24 h between each sequential participant. Two participants were dosed per cohort, and up to five participants could be dosed in a single cohort based on achieving target safety and activity levels.

Dose escalation was based on week 4 FVIII activity levels. If FVIII activity remained <2% at week 4, then dose escalation to the next cohort was triggered. If at least one participant achieved a FVIII activity ≥2%, the decision to escalate or expand the cohort was based on FVIII activity at week 14. If week 14 FVIII activity levels ≥30% were not achieved, then escalation to the next cohort was triggered after review with the data monitoring committee. If FVIII levels of ≥30% were observed in at least one of the two participants in a cohort by week 14, then the cohort was expanded.

Per protocol, if participants exhibited a transaminase elevation of threefold compared to baseline values, glucocorticoids were administered at a starting dose of 60 mg for 4 weeks, followed by a tapering schedule based on twice-weekly AST, ALT, and FVIII activity values. After a protocol amendment, the fourth participant received prednisolone on a prophylactic schedule starting at 60 mg 8 days after the study drug was administered for 4 weeks, and subsequently tapered over 12 weeks. If transaminase elevation occurred again or loss of FVIII expression occurred, prednisolone was re-escalated. Prednisolone dosing and tapering schedule was based on autoimmune hepatitis consensus recommendations.[13]

Safety and dose determination were the primary endpoints of this phase 1/2 trial. Adverse events (AEs) were collected throughout the study and 5-year follow-up period. The CTCAE grading system was used for AE and serious (S)AE collection. Vector shedding was collected as per previous protocols.[11] Efficacy was based on assessments of circulating FVIII activity by one stage assay, FVIII antigen levels, annualized bleeding rates (ABRs), and the use of exogenous FVIII. Three ascending dose cohorts were planned (2×10^{12}, 6×10^{12} and 1.2×10^{13} capsid particles (cp)/kg). Capsid particles were used as dosing units based on internal release assay performance and alignment with regulators. Dose ranges were similar to the more conventional vector genomes per kilogram (vg/kg) values, with an approximate correlation of 1:1. The transgene used in this study was codon-optimized and CpG-reduced by >90% based on prior observations linking CpG motifs with innate immune signalling and subsequent loss of transgene expression.[11] This study was approved by local ethics committees, and all participants provided informed consent.

Neutralizing antibody assays based on in vitro transduction inhibition were performed as previously described.[11,14] Briefly, Serial two-fold dilutions of participant serum were mixed 1:1 with AAV-luciferase and incubated for 2 h at 37°C and then used to infect Huh7 in tissue culture. Following 24 h, luciferin was added, and luciferase activity was quantified by luminometer. The highest dilution of the participant's serum that resulted in inhibition of ≥50% of luciferase activity compared with control was recorded as the NAb titter.

Cell mediated immunity was measured using the ELISpot assay as previously described.[11,14] IFN-γ ELISpot assays for AAV and FVIII-BDD antigen T-cell responses were evaluated using peripheral blood mononuclear cells (PBMCs). A library of 15-mer peptides overlapping by 10 amino acids in sequence was generated to span the entire proteins of interest were organized into three pools. Plates were coated with human IFN-γ coating antibody in sterile PBS, washed, and blocked with complete media. Fresh PBMCs from study participants were adjusted to a concentration of 2×10 cells/mL in lymphocyte culture medium and added to wells. After 18–24 h of stimulation at 37°C, plates were washed and incubated with human anti-IFN-γ horseradish peroxidase (HRP) followed by incubation with Avidin-HRP and subsequent incubation with AEC chromogenic reagent. Human IFN-γ activation counts were quantified using the AID ELISpot Reader. ELISpot sample collection occurred at screening, weekly for weeks 1–2, twice weekly from weeks 3–14, weekly from weeks 15–8, at months 5, 6, 12 and at the end of study.

2.3 | Bulk mRNA transcriptomics analysis

To investigate loss of FVIII expression, bulk mRNA transcriptomic analysis was performed on PBMCs. Whole blood samples from three consented participants were collected at pre-infusion, 8 h post-infusion, study weeks 1–14, and months 4, 5, 6, 9 and 12. Healthy volunteer controls samples were purchased from StemCell Technologies. Whole blood samples were collected in PAXGene tubes and stored at −80°C. Total RNA was extracted with the miRNA Mini Kit. Preparation included total RNA extraction, depletion of haemoglobin mRNA, and construction of bulk mRNAseq libraries followed by sequencing. Equal amounts of six individually indexed cDNA libraries were pooled for clustering in an Illumina cBot system flow cell at a concentration of 8 pM using Illumina's TruSeq SR Cluster Kit V3 and sequenced for 100 cycles using a TruSeq SBS kit on the Illumina HiSeq system. Each sample generated approximately 50 million sequencing reads. Sequencing reads were demultiplexed and exported to fastq files using CASAVA 1.8 software. Data analysis was performed using OmicSoft ArraySuite software, version 10.2.3.10 and R, version 3.6.1. Reads were aligned to the reference genome using the OSA4 algorithm in OmicSoft. Expressed genes were filtered using default parameters in filterByExpr[15] and counts normalized using the trimmed mean of M (TMM), both methods in edgeR (version 3.26.8).[16]

The TMM for the expressed genes were further used for Principal Com-ponent Analysis (PCA) using the PCAtools library (version 2.6.0; removing lower 1% of genes by variance) to check the overall variance of each sample. Pathways were enriched for differentially expressed genes based on literature review of immune responses to AAV injections in vivo. Genes involved in each pathway were curated according to the IPA software package (Qiagen). Analysis was performed comparing the time course of each participant and comparing participants against healthy controls. As this was a safety analysis driven by a specific hypothesis related to immune reactions described against AAV, no formal pathway enrichment analysis was conducted. Gene clustering was performed using custom-written R programs (R code team Version 3.3.2). Gene clusters were visualized in GraphPad Prism 8.2.1 (Figure S1).

Additional computational and genome wide association analyses were conducted to investigate potential risk factors of hypophosphatemia including calculation of a polygenic risk score (see Supplemental Methods).

Study sample size was chosen to provide sufficient evidence of safety and exploration of signs of efficacy for this indication and was not based on formal statistical considerations. Descriptive statistical analyses were performed. Continuous variables were summarized using mean, standard deviation, maximum, minimum, median, and other percentiles as appropriate. Categorical variables including adverse events were summarized using frequency counts and percentages.

3 | RESULTS

3.1 | Participants

Between 2017 and 2021, four participants were enrolled and received an intravenous infusion of TAK-754 in the first two dose cohorts (Figure 1B–E). All four participants were 18 or older and receiving prophylaxis with FVIII concentrates. Baseline ABRs were 0–2. No participants were positive for cross-reactive material (CRIM). Two participants had intron 22 inversion mutations, one participant had a Gln429Ter exon 9 mutation, and one had an Ala1877Pro exon 17 mutation. No participant was positive for HIV, active viral hepatitis B or C, or autoimmune hepatitis.

3.2 | Primary efficacy

After dosing, peak FVIII activities of 3.8% and 11% (cohort 1) and 54.7% and 69.4% (cohort 2) were observed between weeks 4 and 9 (Figure 1B–E). All participants developed minor transaminase elevations and received corticosteroids for periods of 13 –36 weeks.

Initial tapering of glucocorticoids was initiated per protocol as described above but was re-escalated in the setting of either increasing transaminase levels or a decline in FVIII activity. Participant four received corticosteroid prophylaxis beginning 8 days after TAK-754 infusion after a protocol amendment, as described above (Figure 1E). Although each participant experienced temporary protection from

TABLE 1 Treatment emergent adverse events.

TEAE Total	71		
TAK 754 Related AEs	**N**	**Non-severe**	**Severe**
Total	10	8	2
Transaminitis	5	5	0
Asthenia	1	0	1
Hypophosphatemia*	4	3	1
Glucocorticoid-related AEs	**N**	**Non-severe**	**Severe**
Total	14	14	0
Irritability	1	1	0
Insomnia	1	1	0
High blood pressure	1	1	0
Tachycardia	1	1	0
Dermatitis acneiform	1	1	0
Weight gain	1	1	0
Vitamin D deficiency	1	1	0
Hyperphagia	1	1	0
Somnolence	1	1	0
Increased appetite	1	1	0
Night sweats	1	1	0
Rash	2	2	0
Erythema	1	1	0

Abbreviations: AE, adverse evetn; SAE, serious adverse event; TEAE, treatment emergent adverse event;
*Hypophosphatemia SAE occurred in one participant at multiple study visits. Classified as both an AE and SAE at different time points during the study.
Data as of November 3, 2022.

bleeding and was able to discontinue FVIII replacement, sustained expression was not observed, and enrolment was discontinued prior to dosing in the third cohort. All participants have resumed therapeutic haemostatic treatments.

3.3 | Safety

Transient transaminase elevation and glucocorticoid-related adverse events constituted the majority of adverse events (Table 1). No infusion reactions, FVIII inhibitors, thrombosis, or malignancies were observed during the study period. All participants underwent annual screening with liver ultrasound and serum alpha fetoprotein (AFP). No significant pathology was seen on repeated ultrasounds, and AFP levels remained within the normal ranges of the local lab in all participants.

One serious adverse event of asymptomatic hypophosphatemia was reported 4 weeks after infusion of the study drug. This participant required inpatient monitoring on multiple occasions and received both oral and intravenous phosphate supplementation (Figure 2A). Importantly, hypophosphatemia persisted despite tapering from prednisolone. Clinical evaluation did not yield an underlying diagnosis of hyperparathyroidism, bone disease, nutritional deficiency, intestinal malabsorption or genetic risk of hypophosphatemia (Figure 2B). Additional computational and genome wide association analyses were conducted to investigate potential risk factors of hypophosphatemia. No supporting evidence linking this adverse event to the drug's mechanism of action (FVIII expression), modality (AAV gene therapy), co-medication (e.g. prednisolone) or to know genetic factors was found (Figure 2B and Supplemental Methods).

To evaluate an undiagnosed genetic predisposition to hypophosphatemia, a polygenic risk score (PRS) was calculated using data from the United Kingdom Biobank database (UKBB) as described in Supplemental Methods. Using 107,445 variants associated with the blood phosphate levels by genome wide association studies (GWAS), the risk score was created and trained on a database of 316,973 unrelated UKBB samples with known blood phosphate levels. Participant 4's PRS was lower than PRS of controls and was not the sole explanatory factor for the occurrence of the hypophosphatemia adverse event, effectively ruling out an undiagnosed genetic disorder (Figure 2C).

3.4 | Immunogenicity investigations

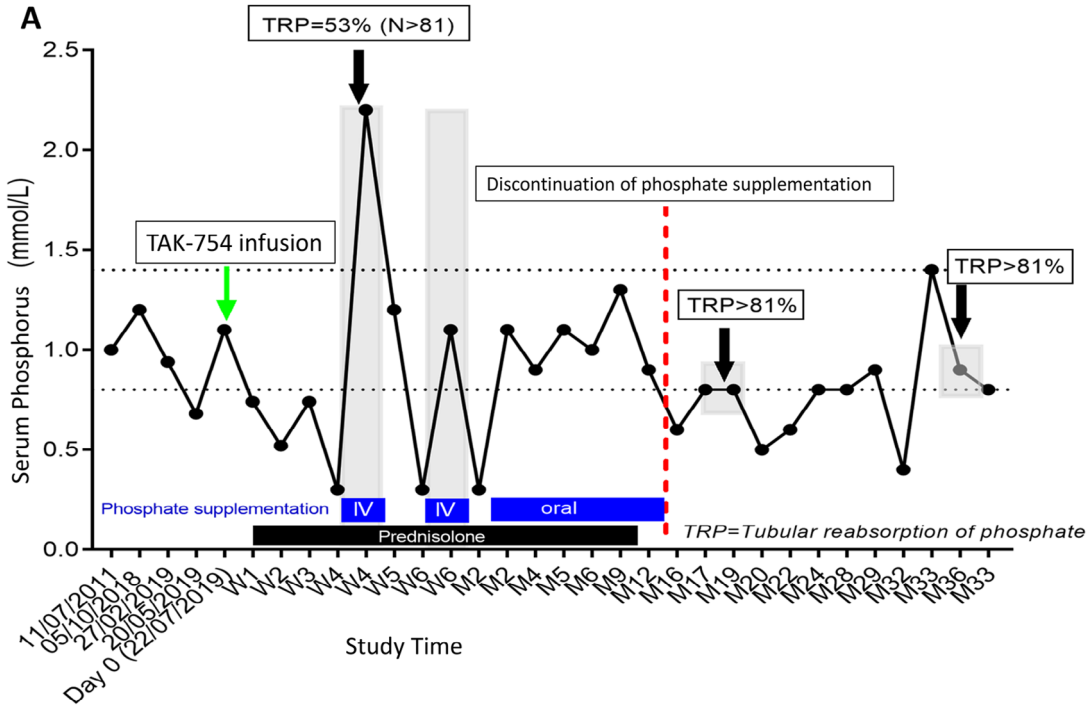
Although ELISpot assays did not correspond with loss of FVIII transgene expression or transaminase elevation in this study, positive responses against the AAV capsid were detected at various time points for all four participants (Figure S2). Serum cytokine analysis of IL-6 and TNF-α performed at the time of TAK-754 infusion remained within the normal ranges for all participants (Table S1).

3.5 | Transcriptomics

To evaluate immune mediated loss of FVIII expression, a transcriptomics analysis was conducted.

RNA Seq bulk mRNA transcriptomics were analysed for activation of MyD88-TLR, anti-viral cytokine and innate immunity signals, canonical and non-canonical NF-kB signals, dendritic cells, STING-C-GAS and T cell populations (Figure S3A–F).

- **MyD88-TLR**: Within 2 h, a transient increase in type I IFNs, TLR9, MyD88, and TNF-α RNA levels were observed that returned to baseline by 6 h. A slight elevation of TLR9 was observed in participants 4 and 2, and both TNF and IRF7 were elevated all three participants 8 h post-AAV infusion. However, no induction of a type I IFN response was observed. No upregulation of TLRs 1-8 or IL-6 proinflammatory genes was observed.
- **Anti-viral cytokines**: A slight elevation of CCL5 was observed in participants 3 and 4 at 8 h and 1 day after AAV infusion. No significant changes in expression of IFNA2, IFNA4, IFNA5, IFNA6, IFNB1, IFNL3 or IL22 was observed.
- **NF-kB**: No elevations of p52, IFNs or other cytokines were observed.



B

Non-genetic factors		Association with Hypophosphatemia
Target	Factor VIII	1/23 Drug Candidates reported hypophosphatemia
Modality	AAV Gene Therapy	1/94 Gene Therapies reported hypophosphatemia
Comedications	Prednisone & Prednisolone	<0.1% FAERS Reporting Frequency Hypophosphatemia persisted following withdrawal
Genetic factors		
Single genes*	Disease-causing mutations in high-penetrance hypophosphatemia genes	PHEX - No pathogenic mutation identified FGF23 - No pathogenic mutation identified DMP1 - No pathogenic mutation identified SLC34A3 - No pathogenic mutation identified ENPP1 - No pathogenic mutation identified FAM20C - No pathogenic mutation identified
Polygenic risk	Phosphate Polygenic Risk Score (PRS)	35% of Phosphate excursions are explained by PRS 10th Percentile for PRS. No significant risk predicted.

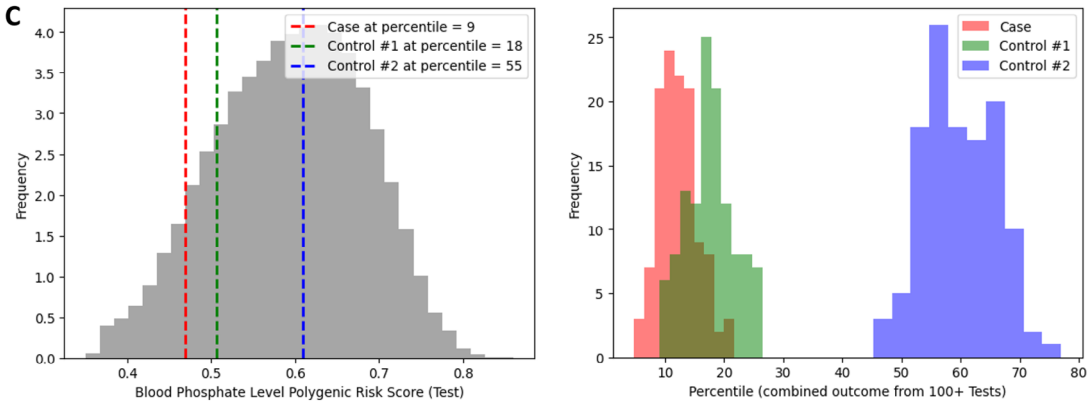


FIGURE 2 (A) Participant 4 hypophosphatemia time course. (B) Participant 4 hypophosphatemia risk factor analysis. (C) Participant 4's polygenic risk score (PRS) compared to both controls and individuals from the UK Biobank (UKBB). On the left side, histogram depicting the PRS for blood phosphate levels in a typical test (refer to Supplementary Methods for details). The test included 20% of unrelated UKBB participants, along with Participant 4 from the TAK-754 trial and two controls, whose PRS percentiles are color-coded. Participant 4's PRS percentile falls

- **Dendritic cells**: A slight elevation of FCER1G was observed in participants 4 and 2 at 1 week post infusion. Small increases in CD83 levels were observed in all three participants 8 h after infusion but were not significantly different from healthy controls.
- **STING-C-GAS sensing pathway**: Mild elevation of IL1B was observed in participants 2 and 3 at 8 h and 1 day post infusion. Slight elevations of IFNGR2 and ISG15 were observed within the first 3 weeks in all patients.
- **T cell marker expression**: A slight elevation of CD4 expression was observed 8 h and 1 day post AAV infusion in participants 3 and 4 but was not significantly different from healthy controls.

4 | DISCUSSION

TAK-754 (BAX888) was based on an AAV8 capsid with a B-domain deleted codon-optimized FVIII transgene under the control of a liver specific promoter. Despite significant reduction in CpG content, loss of transgene expression was observed in all participants and could not be mitigated with early initiation or prophylactic use of high dose glucocorticoids. While the investigational product was well-tolerated, the four study participants have resumed haemostatic treatments and enrolment has been discontinued. The cause of FVIII expression decline in this study remains unclear. No participants were receiving hepatotoxic medication, and all participants had adequate liver function without evidence of viral or autoimmune hepatitis.

Durable multi-year expression of peak FVIII activity remains a significant challenge in haemophilia A gene therapy to date.[4,5,7,17] Study participants achieved a robust elevation in FVIII activity in the second dose cohort accompanied by short term protection from bleeding. However, transgene expression was not sustained. The kinetics of FVIII expression in this study differ from other trials in some respects. Factor VIII activity did not exceed supraphysiologic levels upon initial transduction and loss of FVIII expression was essentially absolute by the end of the first year irrespective of glucocorticoid use. In some gene therapy studies, supraphysiologic FVIII activity is followed by loss of peak expression within the first 12 months, followed by a slower decline.[5,6,18] However, other studies have shown a lower initial peak FVIII expression followed by relatively stable FVIII expression after the first year, usually in the mild haemophilia range (FVIII activity 5%–30%).[7–9] Significant reductions in bleeding and haemostatic treatments have been observed in all trials, confirming bleed protection does not require full normalization of FVIII circulating activity after gene therapy treatment.

The immunogenicity of the gene therapy components (capsid and transgene), cell mediated immunity targeting hepatocytes, activation of an unfolded protein response from endoplasmic reticulum stress, and transcriptional gene silencing have all been proposed as contributing to loss of vector derived FVIII expression.[17,19] The biphasic pattern of FVIII loss observed in some trials has been hypothesized to be related to a shift in either the cell population expressing the transgene or a shift in cellular expression from the episome to an integrated functional transgene. Cell mediated loss of transduced hepatocytes is recognized to occur and lead to loss of transgene expression.[20,21] Whether hepatocytes with higher vector copy numbers (VCNs) are lost early in the post-treatment period while cells with lower VCNs are less vulnerable to cell mediated immunity remains unclear. Similarly, episomal stability impacts transgene expression, and it has been proposed that long term expression may be the result of integration of full-length transgenes into the host genome. Greig et al have demonstrated that gene expression with the AAV delivery platform in non-human primates can recapitulate these clinical trial observations, where expression is initially driven by episomal translation and then by integrated transgenes.[22] However, clinical and in vivo studies of AAV have also demonstrated the persistence of episomes but only low levels of transgene integration.[17,23] Furthermore, Integration of full-length vector transgenes was not observed in long term canine studies, confirming that sustained episomally driven FVIII expression for over a decade is possible in a biologic context.[24] The persistence of episomes is also in line with the patient biopsy data reported by Fong and colleagues.[17] It should be noted that the current study did not achieve a supraphysiologic peak FVIII expression in any participants, thus whether a higher dose of TAK-754 would have achieved a higher peak FVIII activity expression pattern or prolonged steady state expression remains unanswered.

The immunogenicity of the TAK-754 transgene was potentially a contributing factor to loss of expression, however a large amount of preclinical optimization and protocol design features were implemented to avoid innate triggers of immunogenicity. Codon optimization of TAK-754 resulted in >90% reduction in CpG motifs, the presumptive cause of loss of expression in the BAX 335 trial.[11] While the contribution of the remaining CpG motifs cannot be ruled out, a robust innate immune response seems unlikely. Transaminase elevation and loss of vector expression is driven by multiple upstream factors. The loss of transgene expression may have the same observable presentation, but different underlying mechanisms compared to the BAX 335 trial. Indeed, clinical trials have demonstrated significant differences in patterns of FVIII and factor IX (FIX) expression[25] that are likely related to the different properties of the coagulation factors.

slightly below −1 standard deviation, while both controls fall within ±1 standard deviation of the calculated predisposition index. On the right side, the distribution of PRS percentiles for Participant 4 (the case) and the controls, estimated through over 100 experiments. In each of these experiments, the UKBB dataset was randomly split into training and testing sets at an 80/20 ratio. The distribution of the PRS percentiles for the case is significantly lower ($p < 1e^{-5}$; Wilcox test) when compared to the control percentiles. *Genetic analysis on single genes was performed by local laboratory using an NGS panel. W: (study) week; UKBB: United Kingdom Biobank, TRP: Tubular Reabsorption of Phosphate value calculated as: 1-[(urine phosphorus/serum phosphorus) X (serum creatinine/urine creatinine)]; FAERS (FDA Adverse Event Reporting System).

Immunosuppression with high dose glucocorticoids was incorporated into the study protocol but had little discernible effect on the loss of expression, consistent with many clinical observations in the field.[4,5,7,26] Participants in this study and others have received multiple courses of glucocorticoids as transaminase expression and FVIII expression change.[5] We did observe a mild increase in FVIII activity in some participants after the escalation of glucocorticoids. An additional loss of FVIII expression upon discontinuing glucocorticoids has been observed by others. For example, the SPK-8011 multi-year follow-up showed a loss of FVIII expression as some participants were weaned from glucocorticoids, and this is attributed to a glucocorticoid responsive element in the promoter.[7] TAK-754's promoter sequence does not contain a steroid-responsive element.

Zhang et al. evaluated the effect of chronic glucocorticoid treatment after valoctocogene roxaparvovec exposure in a murine model of haemophilia A.[27] Daily prednisolone exposure for 13 weeks did not alter FVIII DNA, RNA or expression in the liver, or protein distribution in the liver. In the absence of a steroid responsive promoter, glucocorticoid exposure did not impact FVIII expression kinetics.

However, chronic blockade of cell mediated immunity can prevent loss of transgene expression. Butterfield and colleagues observed that FVIII expression loss can be blocked by IL-15 blockade in vivo by preventing T and NK cell activation.[28] Importantly, transgene expression could be preserved specifically by CD8+T cell suppression without altering mRNA transgene or vector copy number in the hepatocytes. Furthermore, the use of dexamethasone in mice has resulted in transient increase in transgene expression that may be related to immunomodulatory cytokines as opposed to a direct effect on transcription.[29] More research is needed to understand this phenomenon, although long term immunosuppression with glucocorticoids or other therapies is not likely to be acceptable in the haemophilia population.

Widely used conventional markers of cell mediated immunity (ELISpot and transaminase elevation) did not correlate with loss of expression in this study, and this limitation has been observed by others.[26] We undertook an exploratory transcriptomics analysis to determine if immunogenicity signals could be detected in peripheral blood. No significant changes in NK cells, NF-kB, IL-6, TLRs 1–8, dendritic cell activity, or T cell pathway signals were observed. Small transient increases in TLR9, TNF-α, CCL5, and IRF7 signals occurred 8 h after infusion without activating a Type 1 IFN response or any clinical observations. These responses are likely associated with transient AAV infusion.

While we did not detect a significant immune response in circulating cells, bulk RNA Seq is limited compared to single cell transcriptomics and cannot identify discrete cell populations. However, the PBMCs are enriched in lymphocytes and are a source of immunogenic gene expression. Recently, Xue et al used a more sensitive single cell RNA transcriptomics analysis in a trial of haemophilia B patients treated with a FIX gene therapy and a novel AAV capsid.[30] With this approach, the investigators were able to demonstrate the effect of glucocorticoid prophylaxis on reducing cytotoxic T cells and antigen presenting cells. Participants with elevated markers of cell mediated immunity also had suboptimal responses to FIX gene therapy. To date, clinical trials have not identified reliable assays to predict immunogenicity or loss of FVIII expression and are limited to detection of a signal in peripheral blood. Our observations here and by others would suggest the absence of a significant immunologic response in circulation. Therefore, current peripheral blood biomarkers have limited value for clinical decision-making and are subject to high levels of intra-patient variability. AAV has a relatively low immunogenicity profile in peripheral circulation compared to a tissue-resident cell response and in theory highlights the value of assaying the target tissue (hepatocytes) directly.[31] To date, this type of invasive procedure has not been widely conducted and is not likely to be acceptable for routine use. While the United States Medical and Scientific Advisory Counsil (MASAC) suggests that liver biopsies could be performed in trials on a voluntary basis to inform future studies,[32] it will be important to identify better circulating biomarkers of immunogenicity that predict loss of transgene expression in haemophilia A. Future studies with single cell RNA transcriptomics may help in this effort.

Alternative explanations for the loss of FVIII expression include an exaggerated unfolded protein response against the modified FVIII-BDD, which has been observed in some preclinical models,[33] but not in human liver biopsies.[17] No liver biopsies were performed in this clinical study, and the current evidence does not suggest that the unfolded protein response plays a major role in loss of expression of FVIII. Individual patient-specific responses may be determined by alterations in transcriptional pathway components related to the transgene, promoter, and histone acetylation, all of which require further study.[17] The role of epigenetic changes has recently been identified as a determinant of interspecies variability in gene expression and could play an as-yet uncharacterized role in interindividual variability in clinical trials.[34]

One unusual adverse event (severe hypophosphatemia) was observed in this study. Although this is not a recognized side effect of AAV gene therapies, it has been observed in at least one other trial in a rare metabolic disease.[35] The participant in our study had a protracted recurrent course of hypophosphatemia with the need for intravenous phosphate administration despite the absence of known genetic and clinical risk factors. A polygenic risk score, used to describe the contributions of multiple genes to ascertain risk, did not suggest that this participant was at higher risk for hypophosphatemia compared to a reference control population. The pathophysiology of acute liver disease has been associated with significant hypophosphatemia and renal phosphorus loss via the proximal convoluted tubules,[36] and although this participant's transaminase elevation did not approach the threshold for acute liver failure, AAV exposure at high titters remains a source of hepatic inflammation. The hepatic regulation of phosphate is an area of active research and our observations highlight the consideration of phosphate monitoring after AAV treatment.

After reports of a case of hepatocellular carcinoma in another study, this protocol was amended to include annual liver ultrasounds and serum AFP levels.[37] These studies remained normal in all participants during multi-year follow-up.

There are now several marketed haemophilia gene therapy products, despite a lack of clear understanding of many of the observations from this study across the field. It is encouraging that many haemophilia patients have achieved long term bleeding protection after receiving AAV-based gene therapies, but the factors associated with poor responses require more study. In addition, despite a very favourable safety profile to-date, a full understanding of therapeutic AAV exposure remains incomplete. Long term post-marketing experience and follow-up of trial participants will be key to understanding durability, safety and long-term efficacy that will inform the next generation of gene therapies for haemophilia A.

AUTHOR CONTRIBUTIONS

John Chapin, Mila Ayash-Rashkovsky, Kavitha Rajavel, Jon Kenniston, Matt Wagoner, Qin Wang, Caterina Maggiore, Dorothee Diogo, and Marcin von Grotthuss conducted the study, performed data analysis, and drafted the manuscript. Antoine Rauch and Sophie Susen provided additional analysis for participant 4 adverse event evaluation. All authors analysed data and revised the manuscript.

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CONFLICT OF INTEREST STATEMENT

J.C., M.A.R., K.R., M.W., J.K., D.D., M.G., M.P.W., Q.W. are employees and shareholders of Takeda Pharmaceuticals, Ltd. C.M. is an employee of IQIVIA. M.T.A.R. has received honoraria for speaking engagements or consulting or research funds from Takeda, Bayer, CSL Behring, Grifols, Novo Nordisk, Sobi, Octapharma, Amgen, Novartis, and Pfizer. F.J.L.J. has participated as speaker, in advisory boards and sponsored symposia, with Amgen, Bayer, CSL Behring, Leo Pharma, Novartis, Novo Nordisk, Octapharma, Pfizer, Roche, Sanofi, Sobi, and Takeda. S.S. reports research support from CorWave, Roche-Chugai, Stago; is a trial investigator for Biomarin, Bioverativ, CSL Behring, LFB, Sanofi, Shire/Takeda, Siemens, Healthiners, Sanofi and Sobi; has served as a speaker or advisor for CSL Behring, LFB, Novo-Nordisk, Roche, Siemens, SobiHonoraria, Biomarin,Pfizer, Sanofi, Stago, and Takeda. AR, MEMC report no conflict of interest.

DATA AVAILABILITY STATEMENT

Takeda does not plan to share data supporting the results reported in this article because there is a reasonable likelihood that individual patients could be re-identified due to the very limited (small) study population. Data from UK biobank cannot be shared openly and are subject to UK Biobank ethical approval. Further information about applying for UK Biobank data access can be obtained from the UK Biobank website (https://www.ukbiobank.ac.uk) or by emailing UK Biobank (ukbiobank@ukbiobank.ac.uk).

ETHICS STATEMENT

This study was performed in accordance with the Declaration of Helsinki and was approved by the local ethics committees.

DISCLOSURES

J.C., M.A.R., J.K., M.W., D.D., M.G. and K.R. are current or former employees and shareholders of Takeda Ltd.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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