Received: 11 August 2021 | Revised: 5 March 2022 | Accepted: 13 March 2022

DOI: 10.1002/rth2.12705

ORIGINAL ARTICLE

Seroprevalence to adeno-associated virus type 6 in people with hemophilia B from a UK adult cohort

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Funding information
Sangamo Therapeutics

Handling Editor: Dr Johnny Mahlangu

Abstract

Background: Gene therapy shows promise as a potential "cure" for hemophilia A and B. Adeno-associated virus (AAV) vectors are the leading platform to deliver modified genetic code of factor VIII or IX to the liver effecting endogenous production. Patient exposure to wild-type AAV leads to the formation of neutralizing factors, which can prevent successful transduction. It is thus important to establish the seroprevalence of the AAV serotypes in people with hemophilia to aid prediction of successful gene transfer. The seroprevalence of AAV6 in UK people with hemophilia B is not yet reported.

Objectives: We studied the prevalence of anti-AAV6 neutralizing factors in UK people with hemophilia B ($n = 49$). We collected data on people's hepatitis C exposure and treatment with plasma-derived factor IX (FIX) to identify if there was correlation with AAV6 exposure.

Methods: Serum samples and patient data were collected from 49 people with hemophilia B registered at UK hemophilia comprehensive care centers. The samples were tested for neutralizing factors to AAV6 using a cell-based transduction inhibition assay.

Results: Thirty-one percent of patients had serum neutralization against AAV6. There was no correlation between AAV6 seropositivity and previous treatment with plasma-derived FIX products or hepatitis C exposure.

Conclusion: Based on limited data, there is no evidence of association between the presence of AAV6 neutralizing factors in people with hemophilia B and exposure to contaminated plasma derivatives. The frequency of AAV6 neutralizing factors in our hemophilia B cohort is similar to UK people with hemophilia A and non-hemophilia populations.

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KEYWORDS
AAV, AAV6, adeno-associated virus, gene therapy, hemophilia B, seroprevalence

Essentials

- Adeno-associated viruses (AAVs) are used as vectors for gene therapy to treat haemophilia.
- We tested UK people with hemophilia B for immunity against AAV6.
- Thirty-one percent of people with hemophilia B had neutralizing factors against AAV6.
- Immunity to AAV6 may make people with hemophilia B ineligible for gene therapy using this vector.

1 | INTRODUCTION

Hemophilia B is an X chromosome–linked bleeding disorder caused by a mutation in the gene for factor IX (FIX), an essential protein in the coagulation cascade. Severe hemophilia B is defined by FIX levels <1% of the normal value, and people treated inadequately experience spontaneous and recurrent intra-articular bleeding that leads to painful and disabling chronic joint arthropathy. The current gold-standard treatment is replacement of FIX with regular intravenous infusions of recombinant or plasma-derived FIX. The goal of these infusions is to prevent all spontaneous bleeding by maintaining FIX levels >1 to 3 IU/dL.[1] Additional FIX infusions are administered to treat active bleeding or prevent bleeding in high-risk situations such as surgery or head trauma. Factor infusions can be problematic due to the need for regular venous access, and the individual tailoring of prophylaxis regimens to minimize bleeding risk related to activity can be challenging. A major risk of treatment with exogenous FIX is <5% of people with hemophilia B develop antibodies, termed inhibitors, that make treatment ineffective and may cause anaphylaxis.[2,3]

Recently, there have been significant advances in the treatment of hemophilia, and gene therapy in particular has demonstrated remarkable promise in transforming patients' quality of life. Hemophilia B is an attractive target for gene therapy, as it is a monogenic disease and the laboratory and clinical responses are straightforward to measure through FIX levels and documentation of bleeding episodes, respectively. It has the potential to eliminate spontaneous bleeding, as only a small rise in patients' baseline factor levels has a large impact on the bleeding phenotype.[4] The aim of hemophilia B gene therapy is to maintain a long-term consistent rise in FIX levels after a single infusion of viral vector, thus overcoming the limitations of the current gold standard of care.

Gene therapy for hemophilia is commonly based on using an adeno-associated virus (AAV) vector, such as AAV2, AAV5, AAV6, AAV8, or AAV10, with the human FIX (for hemophilia B) or factor VIII (for hemophilia A) genetic code inserted.[5] These single-stranded DNA viruses are naturally occurring members of the parvovirus family and *Dependovirus* genus. They are unable to replicate autonomously and require a helper virus, such as adenovirus or herpes simplex for replication[6] and have the advantage of being nonpathogenic in humans. AAV has the ability to transduce both dividing and quiescent cells and in the absence of a helper virus can drive long-term transgene expression.[7,8] AAV, like other bloodborne viruses, could potentially be transferred to patients through infusion of blood components and products.[9] An association between seroprevalence to AAV5 and AAV8 and exposure to hepatitis C, through infected blood products, in hemophilia A has been described.[10]

AAV was first used as a gene therapy vector in hemophilia B by injection into skeletal muscle in 1999[11] and has been demonstrated as a safe and effective vector in this genetic disorder.[12] AAV vectors administered intravenously home in on the liver to produce FIX constitutively.[5] However, preformed antibodies against wild-type AAV serotypes can limit this form of therapy,[6] and selecting people according to their positivity is known to lead to successful gene transduction and expression.[12,13]

The prevalence of the AAV6 serotype in the adult population with hemophilia B in the United Kingdom has not yet been reported. As gene therapy clinical trials may potentially use this viral vector platform, it is prerequisite to determine people's eligibility. Our study therefore primarily aimed to assess AAV6 antibody prevalence in the UK hemophilia B community to determine the potential numbers of people for whom this therapy might be effective. As a secondary aim, we sought a link between plasma-derived FIX and hepatitis C exposure and AAV6 immunity.

2 | MATERIALS AND METHODS

We conducted a prospective, observational laboratory study to determine the seroprevalence of AAV6 neutralizing factors and antibodies in people with hemophilia B previously treated with factor X concentrate. The study was reviewed and supported by the Proportionate Review Sub-Committee of the East Midlands–Derby Research Ethics Committee, study number 18/EM/0313.

Serum samples were obtained with informed consent from people registered at six UK hemophilia comprehensive care centers between March and October 2019. The patients were men over the age of 18 years with mild, moderate, or severe hemophilia B, who had previously received treatment with FIX concentrate. People who failed trial screening had no documented use of FIX concentrates, and no eligible people declined to participate in the study. Data were collected on the year of first treatment with FIX concentrate, type of FIX concentrate used for treatment, hepatitis C exposure, and HIV

infection by interviewing participants and reviewing their hospital clinical and laboratory records.

Participant samples were analyzed for preexisting immunity to AAV6 in human serum using a validated cell-based transduction inhibition assay at sample dilution of 1:10. The assay used an AAV6 containing a luciferase reporter gene. Neutralizing substances to AAV6, which include antibodies and serum factors, can reduce the ability of AAV6 in infecting the cell, thereby reducing the expression of luciferase in the cells. Luminescent signals from test samples were normalized to a plate-specific negative control. The presence of neutralizing substances was determined by comparing the normalized response of each sample to a statistically derived assay cutoff. The calculation of the cutoff was consistent with recommended procedures determined using healthy donors with a presumed AAV6-seronegative population.[14,15] Samples with normalized response above the cutoff were determined to be negative for the presence of neutralizing substances to AAV6.

2.1 | Statistical analysis

The participants were placed into two groups according to the AAV6 phenotypes. Continuous variables were provided as mean and range. Categorical variables were outlined in frequencies and percentages. Pearson's chi-square test or Fisher's exact test, where appropriate, was used to analyze the significance of the difference in categorical variables. Correlation coefficient and the significance were evaluated with Spearman's rho test. A p value of <0.05 was considered statistically significant. Statistical analyses were conducted with R 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria) program.

3 | RESULTS

Forty-nine people with hemophilia B, who had previously been treated with FIX concentrate, were recruited into the study. Results were analyzed on all 49 patients; the descriptive characteristics are presented in Table 1.

Of the 49 patients enrolled, 15 (30.6%) had a normalized response below the cutoff in AAV6 serum neutralization assay (p value < 0.34) and thus had AAV6 neutralizing substances present. The median age of AAV6-seropositive participants was 47 years (19-69 years), and the median age of AAV6-seronegative patients was 40 years (19-71 years). Detailed analyses of both AAV6-seropositive and -seronegative groups are shown in Table 2.

All participants in this study had previously been treated with plasma-derived and/or recombinant FIX. The first year of treatment with FIX was reported in 40 (81.6%) participants. For nine participants it was not possible to access hospital records dating as far back as their first treatment, and these participants were unable to recall all childhood treatments. We know, however, that the first recombinant FIX product (BeneFIX) was licensed in the United Kingdom in pediatrics in the year 2000. One participant in the AAV6-seropositive group received their first treatment after the year 2000 and therefore would not have been exposed to plasma-derived FIX. Four participants in that group could not recall the exact date they started treatment. In the AAV6-seronegative group, two participants received treatment after the year 2000, and five participants were unable to recollect the exact year of their first treatment. There was no correlation between exposure to plasma-derived FIX and AAV6 antibodies ($p = 0.99$).

None of the participants had HIV infection. Sixty percent of participants in the AAV6-seropositive group had hepatitis C exposure compared to 50% in the seronegative group and no significant correlation was found between hepatitis C exposure and AAV6 immunity (Spearman's correlation = 0.092; two-tailed p value = 0.53).

TABLE 1 Characteristics of the total haemophilia B cohort

	Median
Age, y (range)	43 (19-71)
Haemophilia B phenotype, *n* (%)	
Severe	27 (55)
Moderate	14 (28)
Mild	8 (16.3)
Exposure to factor IX concentrate, *n* (%)	49 (100)
Treatment with prophylaxis, *n* (%)	27 (55.1)
On-demand treatment, *n* (%)	22 (44.8)
Standard half-life factor IX, *n* (%)	27 (55.1)
Extended half-life factor IX, *n* (%)	21 (42.9)
Trial treatment, *n* (%)	1 (2)
HIV infection, *n* (%)	0 (0)
Hepatitis C antibody negative, *n* (%)	24 (49)
Cleared hepatitis C (antibody positive, RNA negative), *n* (%)	23 (46.9)
Active hepatitis C (antibody positive, RNA positive), *n* (%)	2 (4.1)

4 | DISCUSSION

In the current study, participant samples from those with hemophilia B were tested for for preexisting immunity to AAV6 in human serum using a cell-based transduction inhibition using an AAV6 containing a luciferase reporter gene. We found that 30.6% of participants had neutralizing substances to AAV6.

Recombinant AAV vectors, such as AAV2, AAV5, AAV6, AAV8, or AAV10 are the leading platform for gene therapy in hemophilia. It has been demonstrated that >60% of healthy adults are seropositive for antibodies against AAV from natural infection.[16] The recombinant AAV capsid is a close mimic of wild-type AAV, so antibodies against these AAV subtypes often limit the efficacy of this form of therapy.[6] Even low levels of AAV neutralizing antibodies can limit gene transfer into the liver,[17-19] so it is important to establish the

TABLE 2 Characteristics of participants according to AAV6 immunity status

	AAV6-seropositive participants, *n* (%) *N* = 15 (31%)	AAV6-seronegative participants, *n* (%) *N* = 34 (69%)
Severe hemophilia B	8 (53.3)	19 (55)
Moderate hemophilia B	6 (40)	8 (23.5)
Mild hemophilia B	1 (6.7)	7 (20.6)
Exposure to FIX concentrate	15 (100)	34 (100)
Treated with FIX before year 2000	11 (66.6)	32 (94.1)
Treated with FIX after year 2000	1 (6.6)	2 (5.8)
Unknown date of first treatment with FIX	3 (20)	0
Treatment with FIX prophylaxis	8 (53.3)	19 (55.9)
On demand FIX treatment	7 (46.7%)	15 (44.1%)
Standard half-life FIX	5 (33.3%)	22 (64.7%)
Extended half-life FIX	10 (66.7%)	11 (32.4%)
Trial treatment/ bypassing agents	0	1 (2.9%)
HIV infection	0	0
Hepatitis C negative	6 (40%)	17 (50%)
Cleared hepatitis C	8 (53.3%)	16 (47%)
Active hepatitis C	1 (6.7%)	1 (2.9%)
Total hepatitis C	9 (60%)	17 (50%)

Note: Cleared hepatitis C virus is participants who are hepatitis C virus antibody positive and hepatitis C virus RNA negative. Active hepatitis C is participants who are HCV RNA positive.

Abbreviations: AAV, adeno-associated virus; FIX, exogenous plasma-derived or recombinant factor IX concentrate.

proportion of patients who may not be eligible for gene therapy with each AAV subtype as a potential viral vector. Recently, the uniQure AMT-060 and AMT-061 studies showed the FIX level after administration of AAV-mediated gene therapy was not related to neutralizing antibody titer, using a luciferase-based assay, up to a titer of 678.[20,21] Despite these findings, preexisting neutralizing antibodies are still expected to be exclusion criteria for future gene therapy studies, as preclinical studies have demonstrated that humoral immunity prevents successful signal transduction, and the correlation between antibody titer and effective treatment dosage remains unknown.

The presence of neutralizing activities and antibodies against AAV2, AAV5, and AAV8 has been studied in children with hemophilia A[22] and AAV5 and AAV8 antibodies in adults with hemophilia A.[10] Little has previously been known about the seroprevalence for AAV6 in the hemophilia B population. It has been well established that humoral immunity against various AAV serotypes can be acquired early in life and progressively increase through childhood and adolescence[23] and that there is significant variation in the AAV seroprevalence depending on geographic location.[24] A study in a South African hemophilia B population showed that 82% of analyzed participants had neutralizing antibodies to AAV6, and only 18% would be eligible for AAV6 vector-based gene therapy.[25] A recently conducted study examining global seroprevalence of preexisting immunity against various AAV serotypes in the hemophilia A population showed AAV6 seroprevalence ranging between 31% and 61% in seven different countries, with a level of 35% in the UK hemophilia A population.[26] This was based on an anti-AAV total antibody assay.

Our study demonstrated the presence of serum-neutralizing substances to AAV6 in 31% of UK participants with hemophilia B. Our study indicates that nearly one-third of patients would be not be eligible for participation in AAV6-mediated gene therapy trials with an exclusion criterion of demonstrable AAV6 immunity. All participants had received FIX treatment, and the majority received their first treatment before recombinant products were available. There was no correlation between AAV6 seroprevalence and hepatitis C infection that occurred through contaminated blood products. The rate of AAV6 seroprevalence in our hemophilia B cohort was similar to rates reported in the UK hemophilia A population (35%)[10] as well as US and Northern European healthy adult populations (30%-46%).[16,27] This is consistent with previous comparisons of seroprevalence to AAV2 and AAV8 between the healthy population and people with hemophilia, which have demonstrated no difference.[24]

4.1 | Limitations

As discussed, obtaining accurate historical information on lifelong hemophilia treatments was not possible in all our participants. Due to the age of participants lacking in these data, it is likely the unknown whether the first year of treatment occurred before the year 2000 when only plasma-derived FIX was available, but we could not report data based on this assumption. Data on the ethnicity of participants was available in only four of six of the participating centers, so we are unable to report if our samples are representative of the UK population. It should be taken into account that comparison of data with other studies used a differing methodology in reporting AAV6 seropositivity. The majority of preexisting antibodies against AAV cross react with all AAV serotypes but with different neutralization tiers across serotypes. Since our assay used an AAV6-luciferase as the reporter, the assay does measure neutralization to AAV6 but does not rule out cross reactivity to other serotypes.

5 | CONCLUSION

Gene therapy offers the potential to transform the quality of life of patients with severe hemophilia. Due to preexisting immunity to wild-type AAV, there may be a limit to how many patients will have successful transduction using the various AAV subtypes. Based on our findings, 70% of adults with hemophilia B in the UK would have serological eligibility for gene therapy using a recombinant AAV6 vector. It is likely that the presence of AAV6 neutralizing factors in people with hemophilia B occurs through natural infection rather than through contaminated blood products, so the frequency of immunity is unlikely to differ significantly as a larger proportion of the adult hemophilia population becomes plasma product naïve.

ACKNOWLEDGMENTS

This study was funded by a grant from Sangamo Therapeutics. Sangamo arranged the analysis of the samples - assays for pre-existing immunity to AAV6 in human serum were arranged through Sangamo Therapeutics. The authors thank the patients who participated in the study and the research teams at all the recruiting sites.

RELATIONSHIP DISCLOSURE

All authors stated that they had no interests that might be perceived as posing a conflict or bias. Funding was provided to University Hospital Southampton NHS Foundation Trust, who were the Sponsor in this study. The company did not play a specific role in the research in terms of design, conduct, writing or decision to publish.

AUTHOR CONTRIBUTIONS

SR designed the research. SB and IJ wrote the article. KC analyzed the data. CM coordinated the study. All other authors reviewed and provided expert comments on the article.

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How to cite this article: Boyce S, James I, Rangarajan S, et al. Seroprevalence to adeno-associated virus type 6 in people with hemophilia B from a UK adult cohort. *Res Pract Thromb Haemost*. 2022;6:e12705. doi:10.1002/rth2.12705