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ORIGINAL ARTICLE

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Adenovirus-associated antibodies in UK cohort of hemophilia patients: A seroprevalence study of the presence of adenovirus-associated virus vector–serotypes AAV5 and AAV8 neutralizing activity and antibodies in patients with hemophilia A

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Abstract

Background: Current treatment for severe hemophilia A is replacement of deficient factor. Although replacement therapy has improved life expectancy and quality, limitations include frequent infusions and high costs. Gene therapy is a potential alternative that utilizes an adeno-associated virus (AAV) vector containing the human genetic code for factor 8 (FVIII) that transduces the liver, enabling endogenous production of FVIII. Individuals with preexisting immunity to AAV serotypes may be less likely to benefit from this treatment.

Objectives: This study measured seroprevalence of antibodies to AAV5 and 8 in an UK adult hemophilia A cohort.

Patients/Methods: Patients were recruited from seven hemophilia centres in the UK. Citrated plasma samples from 100 patients were tested for preexisting activities against AAV5 and 8 using AAV transduction inhibition and total antibodies assays.

Results: Twent-one percent of patients had antibodies against AAV5 and 23% had antibodies against AAV8. Twenty-five percent and 38% of patients exhibited inhibitors of AAV5 or AAV8 cellular transduction respectively. Overall seroprevalence using either assay against AAV5 was 30% and against AAV8 was 40% in this cohort of hemophilia A patients. Seropositivity for both AAV5 and AAV8 was seen in 24% of participants.

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Conclusions: Screening for preexisting immunity may be important in identifying patients most likely to benefit from gene therapy. Clinical studies may be needed to evaluate the impact of preexisting immunity on the safety and efficacy of AAV mediated gene therapy.

KEYWORDS
adeno-associated viral vectors, hemophilia A, seroprevalence

Essentials

- Gene therapy is a potential long-term treatment for hemophilia A.
- Hemophilia A patients were recruited in the UK and tested for preexisting immunity to AAV5 and AAV8.
- 21% of patients had antibodies against AAV5 and 23% had antibodies against AAV8.
- 25% of patients had inhibitors to AAV5 and 38% of patients had inhibitors to AAV8.

1 | INTRODUCTION

Current treatment for hemophilia A involves administration of factor concentrates to prevent or treat bleeds. Although replacement of FVIII has improved life expectancy and quality, limitations include frequent infusions at high costs and the risk of inhibitor formation.[1,2] Gene therapy is a potential alternative long-term treatment option that works by producing endogenous FVIII following a single intravenous infusion of a vector containing the appropriate genetic code that has trophism for the liver.[3] This enables the liver to produce relevant factor and even modest increases (plasma level of 2 ng/mL resulting in an increase in activity of 1%) can ameliorate severe forms of the disease.[4] To date, gene therapy has been successfully used to treat hemophilia B[5-9] and recently successful gene transfer has been reported in hemophilia A.[10]

The adeno-associated virus (AAV) serves as a promising gene delivery system as it can transduce dividing and non-dividing cells and has not been associated with any disease.[11-13] Adeno-associated viruses are small, non-enveloped single stranded, DNA viruses belonging to the *Parvoviridae* family and *Dependovirus* genus that cannot replicate autonomously and require a helper virus such as herpes simplex or adeno virus.[14] Preexisting immunity against AAV vectors may represent a major barrier in gene transfer which could potentially result in clearance of the vector before it reaches the target cell.[9,15] The impact of preexisting immunity suggests that screening patients for seroprevalence may help identify those most likely to benefit from gene transfer.

Seroprevalence to different AAV serotypes is measured by either: (a) total antibody binding to the AAV capsid via immunoassay, or (b) detection of inhibitors that neutralize in vitro and in vivo the ability of AAV vectors to transduce.[16] Immunoassay is a capture-based method to detect antibodies capable of binding to the AAV capsid. The AAV capsid or peptide is coated on a plate, plasma or serum added, and antibodies detected with a secondary reagent.[17] In vitro cell-based assays use a reporter AAV vector that is incubated with the test sample before transduction of a cell line. These are amongst the most widely used methods of determining anti-AAV neutralizing factors and the transduction inhibition assay is considered a standard.[18]

With the clinical application of gene therapy using AAV5 and AAV8 in hemophilia,[7,19] this study aimed to measure the prevalence of these serotypes using assays that measure transduction inhibition and total antibody level in the UK hemophilia A population. Secondary aims included measuring differences in the prevalence of AAV5 and AAV8 in those who were exposed to plasma derived products and those who were not. Furthermore, differences in seroprevalence of AAV5 and AAV8 based on human immunodeficiency virus (HIV) and hepatitis C status as well as exposure were also assessed.

2 | MATERIALS AND METHODS

Plasma samples from a total of 101 hemophilia A patients recruited from seven UK hemophilia centers were tested for preexisting neutralizing factors to AAV5 and AAV8 using transduction inhibition (TI) activity and total antibody assay (TAb). Favourable ethical opinion for this study was obtained from the National Research Ethics Committee North West–Liverpool Central, study number 15/NW/0469. The AAV5 assays were developed by the department of Bioanalytical Sciences at Biomarin Pharmaceutical Inc and the AAV 8 assays were developed by Genosafe. The information on HIV and hepatitis C was obtained from historical medical records.

2.1 | AAV5 and AAV8 total antibodies assay for human plasma

Total antibodies against AAV5 were measured in human plasma using a validated sequential bridging electrochemiluminescence (ECL) assay on the MSD platform as described previously.[20] Sample results were expressed as an signal of noise (S/N) value, calculated by dividing sample ECL units by negative control ECL units. Samples that had S/N values >1.15 were considered positive. AAV5 TAb titers

were determined as the reciprocal dilution of plasma samples at the titer cut point, S/N = 1.30. Average sensitivity for measuring antibodies to AAV5 was 4.5 ng/mL.

Total IgG antibodies to AAV8 were measured using a previously published ELISA technique.[18] All samples with a mean optical density of >0.506 were considered and results reported as the reciprocal titer at the cut point. As there are no human anti-AAV8 monoclonal antibodies available, the AAV8 limit of detection was determined to be 18.8 μg/mL using human intravenous immunoglobulins (IVIg) solution.

2.2 | Cell-based AAV5 and AAV8 transduction inhibition titer assay for human plasma

A validated AAV5 TI assay for human plasma samples has been previously described.[20] AAV5 TI titers were determined as the reciprocal dilution of plasma samples at the titer cut point, 44.9% transduction of the negative control. The method used to measure the neutralising effect of AAV8 has also been previously described.[18] AAV8 TI titers were determined as the first reciprocal dilution at which >38.4% inhibition of transduction by comparison with the negative control.

2.3 | Statistical analysis

Continuous variables are presented as median and interquartile range (IQR) and categorical data as frequency and percentages. To evaluate differences between groups, Pearsons's chi-square test was used when the expected cell frequencies were equal to or greater than 5 and Fisher's exact test was used when the expected numbers were less than 5. Univariate analysis was performed to identify significant variables and those significant were used in the multivariate logistic regression analysis to identify the independent predictive variables. *P* values (two-tailed) <0.050 was considered statistically significant. Data were analyzed using SPSS version 23 (IBM, Armonk, New York).

TABLE 1 Demographics of the hemophilia A cohort

	Median (IQR)
Age	38 (27-57)
Weight (kg)	78 (70-88.8)
Baseline FVIII level	No. of patients
<1 IU/dL	60
1-5 IU/dL	14
>5 IU/dL	26
Exposure to plasma products	86
Treatment	
Prophylaxis	45
On-demand	55
HIV positive	6
Hepatitis C exposed	42
Hepatitis C positive	22

3 | RESULTS

Of the 101 patients recruited, one was excluded as the patient was not previously treated with FVIII. Results were analysed for 100 patients and descriptive characteristics of the population are presented in Table 1.

The number of positive and negative patients for AAV5 and AAV8 based on the TI and TAb assays are presented in Table 2. In this study, positivity in either or both assays were used as a measure of seroprevalence. Seropositivity using TI and TAb assays are presented in Table 3.

In patients positive for AAV5 the TI assay titers ranged from <2 to >256 and for the TAb assay the range was 57 to 2246. In AAV8 positive patients TAb assay titers ranged from 125 to 11 949 and for the TI assay the ranges were 3.35 to >256. No significant correlation was observed between TAb and TI titers for AAV5 ($r = 0.68$, $P = 0.064$), TAb and TI titers for AAV8 ($r = 0.62$, $P = 0.070$) and TI titers between AAV5 and AAV8 ($r = 0.56$, $P = 0.087$). A significant correlation ($r = 0.77$, $P = 0.001$) was observed in TAb titers between AAV5 and AAV8 (Figure 1). Relationships between exposure to plasma products, HIV and hepatitis C status, hepatitis C exposure and treatment were assessed, results were available for all 100 patients and are presented in Table 4. Mutlivariate analysis of increasing age and seroprevalence of both AAV5 and AAV8 are presented in Table 5.

4 | DISCUSSION

In the present study, patient samples were tested for inhibitors of transduction and total antibodies for AAV5 and 8. We found that 21% of patients had antibodies to AAV5 and 23% of patients had antibodies to AAV8. Twenty-five percent of patients exhibited inhibitors to AAV5 transduction and 38% of patients had inhibitors of AAV8 transduction. Thirty percent of patients were positive in either assay for AAV5 and 40% for AAV8. Fifteen percent had antibodies against both AAV5 and AAV8, 23% had inhibitors against both capsids, and 24% were positive in both assays for both AAV5 and AAV8.

Previous studies on AAV seroprevalence have indicated that following exposure there is production of antibodies against AAV from all four IgG subclasses which harbor neutralizing properties but other unidentified factors within individuals may also be present.[21]

TABLE 2 Seroprevalence of AAV5 and AAV8 in the United Kindgom based on transduction inhibition and total antibodies assays. Percentage of patients who were positive for both AAV5 and AAV8 as measured by transduction inhibition and total antibodies assays

% Positive patients				
	TAb	TI	Either	Both
AAV 5	21	25	30	16
AAV 8	23	38	40	21
AAV 5 & AAV 8	15	23	24	15

TABLE 3 Comparison (%) between the transduction inhibition and total antibodies assays for AAV5 and AAV8

Total antibody (TAb)/transduction Inhibition (TI) assay	AAV 5 (%)	AAV 8 (%)
TAb− TI−	70	60
TAb+ TI+	16	21
TAb+ TI−	5	2
TAb− TI+	9	17

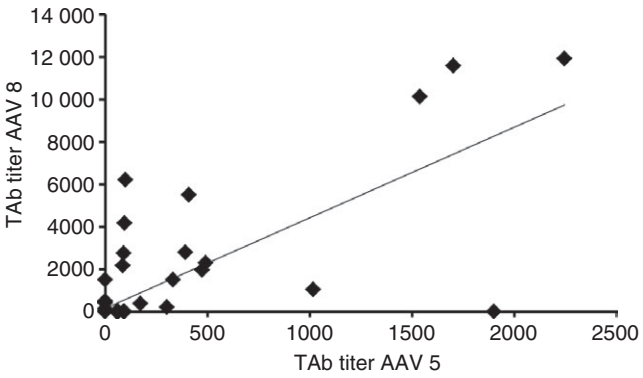

FIGURE 1 Correlation of total antibodies titers of AAV5 and AAV8 in the adult UK hemophilia A population (r = −0.77, P = 0.001)

Therefore, current estimates of seroprevalence encompass measures of antibodies against AAV or other neutralizing factors (Table 6).

Currently, inhibitors of transduction to different AAV serotypes are determined by either in vitro or in vivo methods.[16] Previous studies have demonstrated that several factors impact results including: assay sensitivity based on starting dilution, cell lines used for in vitro transduction, quantity of input AAV vector per cell, temperature, incubation time, volume of human sample used for AAV interaction, reporter transgene, eg, luciferase, LacZ etc., culture duration after addition of AAV transduction and heat inactivation of complement proteins.[5,15,21-43] In vitro assays were used in this study and the cut-off titer values at which patients were considered positive for total antibodies was set at >1.15 for AAV5 and >0.506 for AAV8. Transduction inhibiton positive cut-off titers were 44.9% for AAV5 and 38.4% for AAV8. These cut-off values were determined by statistically analysing sufficient numbers of samples from healthy popluations, ensure distribution was normal by removing outliers and setting the desired rate of false positive results, generally 1%-5%.[18,20] No significant correlations were observed between the TAb and TI titers against either capsid, specifically AAV5 (r = 0.68) or AAV8 (r = 0.62) nor when comparing the inhibitor titer values between AAV5 vs AAV8 (r = 0.56). It is noteworthy that in comparing antibody or TI titers between the two AAVs, differences in the quantity of virus employed in the cell-based assays is likely to result in different sensitivities of the AAV5 vs AAV8 assays and hence should be taken into account. A significant correlation was observed between TAb titers of AAV5 and AAV8 confirming the existence of cross reactivity which has been observed in previous studies.[21,44]

TABLE 4 Relationships of AAV5 and AAV8 seroprevalence with plasma product use, human immunodeficiency virus status and hepatitis C (exposure and status) (% positive)

	Plasma (% positive)	HIV (% positive)	Hepatitis C exposure (% positive)	Hepatitis C status (% positive)
AAV5 TI	28	17	40*	36
AAV5 TAb	22	17	33*	41*
AAV8 TI	41	17	48	50
AAV8 TAb	27*	0	33*	36

*P < 0.05.

TABLE 5 Multivariate analysis of increasing age and seroprevalence of AAV5 and AAV8

	Age <38 y	Age ≥38 y	Number of patients	OR (95% CI)
AAV5 TI				
Negative	47	32	79	0.16 (0.04-0.52)
Positive	4	17	21	
AAV5 TA				
Negative	45	30	75	0.21 (0.08-0.56)
Positive	6	19	25	
AAV8 TI				
Negative	46	31	77	0.16 (0.05-0.52)
Positive	4	17	21	
Negative	38	21	59	0.32 (0.13-0.74)
Positive	12	25	37	

TABLE 6 Current estimates of the seroprevalence of AAV 5 and AAV 8

Authors	Region	n	Population	AAV 5 antibodies (%)	AAV 8 antibodies (%)	AAV 5 neutralizing factors (%)	AAV 8 neutralizing factors (%)
Boutin et al[21]	France	226	Healthy adults	40	38	3.2	19
Calcedo et al[44]	Australia, Europe, Africa, USA,	100	Healthy adults	—	5-32	—	—
Liu et al[45]	China	500	Healthy children and adults	40.2	82	—	—
Liu et al[45]	China	270	HIV-1 infected	—	—	37	—
Li et al[26,43]	Thailand, USA	62	Paediatric hemophilia A	25.8	22.6	—	—
Mimuro et al[47]	Japan	85	Healthy adults	37.6	32.9	—	—
Mimuro et al[47]	Japan	59	Hemophilia	35.6	32.9	—	—
Falese et al[20]	USA	100	Healthy adults	5	—	24	—
Falese et al[20]	USA	24	Hemophilia A	8	—	8	—

A previous study in hemophilia B demonstrated that neutralizing antibodies at titers lower than 1:10 could completely neutralize large vector doses with undetectable transgene expression.[9] Currently, there is a lack of standardization of assays that measure neutralizing activity and total antibodies, therefore there is a variation in cut-off titer values where participants are considered positive. Previous studies set the cut-off positive titer at ≥50%,[21,26,45] which was higher than the positive cut-off values set in this study, and may influence the results. It is also important to note that for previous studies[26,28,29] the cut-off was determined in healthy US donors. In addition, the cut-off threshold for determining seroprevalence may change with geographical location and/or the utilization of patient-specific samples during validation of an assay. It is imperative therefore to standardize measurement of seroprevalence of AAV in hemophilia patients, not only as a single measurement but in a longitudinal study as this may impact the eligibility and outcome of gene therapy.

We report a significant increase in seroprevalence of AAV8 in patients exposed to plasma products. There was also a significant increase in seroprevalence of both AAV5 and AAV8 in patients who were exposed to hepatitis C. Further investigation is required to establish the cause of increased seroprevalence due to plasma exposure and hepatitis C. However, transfusion transmitted infection cannot be discounted.

Seroprevalence was higher with increasing age in this study, which has been observed in previously. Evidence from healthy individuals in Japan showed that AAV infection occurred in childhood and seropositivity subsequently decreased, however there was a second increase of seropositivity after 30 years of age.[46] Similarly, another Japanese study of healthy as well as hemophilia patients observed an increase in seropositivity with age.[47]

4.1 | Limitations

As discussed previously, various factors influence the sensitivity of assays for measuring TAb and TI to AAV and these need to be considered while interpreting results. The number of positive patients differed depending on the type of assay and therefore both should be considered at this stage to screen potential patients for gene therapy. With currently available tests there is a variation in positive cut-off values, therefore there needs to be a focus on standardising these assays. Measurement of AAV antibodies was only done at a single time point and evaluation of varying titer levels over time should be considered for future studies. Divergent methodology of the assays employed to measure AAV5 and AAV8 must be taken into consideration when interpreting results.

5 | CONCLUSION

Despite progression in the current treatment of hemophilia A from plasma derived to recombinant products, natural exposure to AAV occurs at an early age, as shown in seroprevalence studies in both healthy and paediatric hemophilia populations.[20,26,43] Therefore, screening may need to be considered for those most likely to respond to gene therapy with AAV vectors. It is currently unknown whether some level of seropositivity to AAV may be tolerated and clinical studies will need to evaluate the effect of preexisting immunity on the safety and efficacy of AAV mediated gene therapy. Further investigation is required to explore the link between increased seroprevalence in patients exposed to plasma products and Hepatitis C.

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RELATIONSHIP DISCLOSURES

Dr S. Rangarajan is a study investigator, Prof J. Pasi has received grant support, personal fees, and non-financial support and Drs G. Hayes and S. Fong are employees of Biomarin Pharmaceutical Inc. Dr G. Hayes also has a patent null pending. Ms R. Pink and Dr S. Stanford report grants from Biomarin Pharmaceutical Inc. during the conduct of the study. The remaining authors stated that they had no interests which might be perceived as posing a conflict or bias.

AUTHOR CONTRIBUTIONS

Dr S. Rangarajan designed the research. Dr S.N. Stanford wrote the paper. Mr K. Chandrakumaran and S.N. Stanford analyzed the data. Mrs R. Pink coordinated the study. All other authors reviewed and provided expert comments on the paper.

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