Differential Prevalence of Antibodies Against Adeno-Associated Virus in Healthy Children and Patients with Mucopolysaccharidosis III: Perspective for AAV-Mediated Gene Therapy

Haiyan Fu,^{1,4,*} Aaron S. Meadows,¹ Ricardo J. Pineda,¹ Krista L. Kunkler,¹ Kristen V. Truxal,^{1,3,4} Kim L. McBride,²⁻⁴ Kevin M. Flanigan,^{1,4,5} and Douglas M. McCarty^{1,4}

¹Center for Gene Therapy, ²Center for Cardiovascular Research, ³Division of Molecular and Human Genetics, Research Institute at Nationwide Children's Hospital, Columbus, Ohio; Department of ⁴Pediatrics and ⁵Neurology, School of Medicine The Ohio State University, Columbus, Ohio.

Recombinant adeno-associated virus (AAV) vectors are promising gene therapy tools. However, pre-existing antibodies (Abs) to many useful AAV serotypes pose a critical challenge for the translation of gene therapies. As part of AAV gene therapy program for treating mucopolysaccharidosis (MPS) III patients, the seroprevalence profiles of AAV1-9 and rh74 were investigated in MPS IIIA/IIIB patients and in healthy children. Using enzyme-linked immunosorbent assay for α AAV-IgG, significantly higher seroprevalence was observed for AAV1 and AAVrh74 in 2- to 7-year-old MPS III patients than in healthy controls. Seroprevalence for the majority of tested AAV serotypes appears to peak before 8 years of age in MPS III subjects, with the exception of increases in α AAV8 and α AAV9 Abs in 8- to 19-year-old MPS IIIA patients. In contrast, significant increases in seroprevalence were observed for virtually all tested AAV serotypes in 8- to 15-year-old healthy children compared to 2- to 7-year-olds. Co-prevalence and Ab level correlation results followed the previously established divergence-based clade positions of AAV1-9. Interestingly, the individuals positive for α AAVrh74-Abs showed the lowest co-prevalence with Abs for AAV1-9 (22-40%). However, all or nearly all (77-100%) of subjects who were seropositive for any of serotypes 1-9 were also positive for α AAVrh74-IgG. Notably, the majority (78%) of α AAV seropositive individuals were also Ab-positive for one to five of the tested AAV serotypes, mostly with low levels of α AAV-Abs (1:50-100), while a minority (22%) were seropositive for six or more AAV serotypes, mostly with high levels of α AAV-IgG for multiple serotypes. In general, the highest IgG levels were reactive to AAV2, AAV3, and AAVrh74. The data illustrate the complex seroprevalence profiles of AAV1-9 and rh74 in MPS patients and healthy children, indicating the potential association of AAV seroprevalence with age and disease conditions. The broad co-prevalence of Abs for different AAV serotypes reinforces the challenge of pre-existing α AAV-Abs for translating AAV gene therapy to clinical applications, regardless of the vector serotype.

Keywords: AAV, pre-existing Abs, MPS III, seroprevalence

INTRODUCTION

MUCOPOLYSACCHARIDOSIS (MPS) III is a group of four (A, B, C, and D) neuropathic lysosomal storage diseases, each caused by the autosomal recessive defect in a specific lysosomal enzyme that is essential for the stepwise degradation of heparan sulfate (HS) glycosaminoglycans (GAGs).¹ The lack of the specific enzyme activity results in the accumulation of HS-GAGs in cells in virtually all organs. Patients with MPS III appear normal at

birth, but progress to multisystem manifestations, with severe profound neurological disorders, leading to high mortality and premature death.

No effective treatment is currently available for MPS III. However, recombinant adeno-associated virus (rAAV) mediated gene therapy has shown promise for the treatment of MPS IIIA and IIIB.²⁻⁶ The AAV vectors are promising as effective gene delivery tools for long-term transduction in a broad range of tissues. They have displayed efficacy and

*Correspondence: Dr. Haiyan Fu, Center for Gene Therapy, Research Institute at Nationwide Children's Hospital, 700 Children's Drive, Columbus, OH 43205. E-mail: haiyan.fu@nationwidechildrens.org

safety after systemic delivery in numerous preclinical disease models and in clinical trials, particularly for monogenic diseases.^{2,4,7–10} The recognition of the trans-blood–brain barrier (BBB) neurotropic properties of rAAV9 vector^{11,12} have led to significant advancements in AAV gene delivery for diseases with global or broad neuropathies in the central nervous system (CNS), demonstrating promising clinical potential.^{2–4,10,13} These studies have led to the translation of systemic rAAV9 gene delivery to a Phase I clinical trial in patients with type 1 spinal muscular atrophy (SMA1; NCT02122952) and Phase I/II trials in patients with MPS IIIA (NCT02716246, ongoing) and MPS IIIB (to be initiated), and intrathecal gene delivery clinical trials in patients with giant axonal neuropathy (NCT02362438) and Batten disease (CLN6; NCT02725580).

As effective rAAV gene therapy approaches become available for clinical application, pre-existing host humoral immunity against AAV poses critical challenges. While having no known pathogenesis, AAV is widespread in humans, and >90% of the adult population is naturally infected, with a high prevalence of antibodies (Abs) to various AAV serotypes.^{14,15} Although AAV2 is the most prevalent in humans, cross-reactivity among different serotypes reduces the potential utility of rAAV vectors packaged into alternative serotypes.^{14–16} While the presence of neutralizing Abs against specific AAV serotypes has been demonstrated to block the type-specific AAV vector,¹⁷ non-neutralizing anti-AAV Abs can also trigger vector clearance.¹⁸ For rAAV gene therapy clinical trials, the absence of anti-AAV (α AAV) neutralizing Abs is often used as a critical exclusion criteria, though a serum α AAV total immunoglobulin G (IgG) titer <1:50 has also been accepted by the Food and Drug Administration as negative for allowing subject enrollment (NCT02122952, NCT02716246, NCT02725580, NCT02376816, NCT02354781, and NCT01976091).

In support of an ongoing rAAV gene therapy program, serum samples from patients with MPS IIIA or IIIB were compared to healthy children for total IgG against AAV1–9 and rh74 in order to determine the profiles of pre-existing α AAV-Abs in these pediatric populations, and the potential utility of different AAV serotypes as vectors for future gene therapy design.

MATERIALS AND METHODS

Serum samples

Serum samples were obtained from patients with MPS IIIA ($n=24$) and MPS IIIB ($n=14$), who enrolled in a natural history study¹⁹ or a MPS III inflammation study at Nationwide Children's

Hospital (NCH), following informed consent and under Institutional Review Board (IRB)-approved protocols (IRB#13-0330 and IRB#09-00235). All MPS III patient subjects met the criteria, with a confirmed diagnosis of MPS IIIA or MPS IIIB by either the presence of homozygous or compound heterozygous pathogenic variants in the *SGSH* or *NAGLU* genes, or undetectable or significantly reduced *N*-sulphoglucosamine sulphohydrolase (SGSH) or α -*N*-acetylglucosaminidase (NAGLU) enzyme activity in leucocytes or plasma. Blood samples obtained from MPS III patients were processed within the NCH Biopathology Core or in the laboratory of the corresponding author. Serum samples were aliquoted and stored at -80°C on the same day as blood sampling. Control serum samples were obtained from healthy individuals ($n=35$) via BioServe (Beltsville, MD), and the MPS III inflammation study approved by NCH-IRB (IRB#09-00235). Table 1 summarizes the demographic distribution of the study subjects.

Binding enzyme-linked immunosorbent assay

Serum samples were assayed by binding enzyme-linked immunosorbent assay (ELISA) to determine the levels of total IgG against AAV serotypes 1–9 and rh74, following previously published procedures.²⁰ Empty capsid particles of AAV1, 2, 3, 4, 5, 6, 7, 8, and 9 and rh74 were obtained from SAB Tech, Inc. (Philadelphia, PA).

Briefly, 96-well plates were coated with 1×10^{10} particles/mL of empty AAV9 capsids in carbonate coating buffer (antigen-positive [ag^+]) and carbonate coating buffer only (antigen-negative [ag^-]) for each sample. Following incubation overnight at 4°C , the plates were then washed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (pH 7.4) and blocked for 1 h with blocking buffer (5% milk in PBS containing 0.1% Tween 20). Two-fold serial dilution (beginning at 1:50) of serum samples in blocking buffer was added to the plates and incubated at room temperature for 1 h. The

Table 1. Study subjects

Subjects	Age, years ^a	n (M/F)
MPS IIIA	2–7 (4.7 ± 1.5)	16 (6/10)
	8–18 (11.9 ± 4.4)	8 (3/5)
MPS IIIB	2–7 (5.7 ± 2.0)	5 (2/3)
	8–20 (11.6 ± 3.4)	9 (3/6)
Controls	2–7 (4.5 ± 2.0)	18 (9/9)
	8–15 (10.5 ± 2.5)	17 (7/10)

^aAge is shown as mean $\pm$ standard deviation.
MPS, mucopolysaccharidosis.

plates were washed with PBS-T and then incubated with horseradish peroxidase conjugated anti-human IgG (Sigma-Aldrich, St Louis, MO) for 1 h at room temperature. After being washed with PBS-T, the plates were then developed with 3,3',5,5'-tetramethylbenzidine (TMB; Sigma-Aldrich) at room temperature for 5 min. The reaction was stopped by adding 1 M of sulfuric acid. The absorbance was read at 450 nm on a plate reader. Serum total α AAV-IgG levels are expressed as ELISA titer, based on the following calculation: $OD_{450} - ag^+ - OD_{450} - ag^- / OD_{450} - ag^-$. Values ≥ 2 were considered antibody-positive.

Data analyses

Data were analyzed using an unpaired Student's *t*-test, Fisher's exact test, and Pearson's correlation coefficient. Significance was defined as $p \leq 0.05$.

RESULTS

In this study, ELISA was performed to analyze serum samples for total IgG specific for different AAV serotypes to determine the prevalence and levels of α AAV-Abs in patients with MPS IIIA (aged 2–18 years; $n = 24$) or MPS IIIB (aged 2–20 years; $n = 14$) and healthy controls (aged 2–15 years; $n = 35$) (Table 1 and Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/humc). Serum α AAV-IgG titer $\geq 1:50$ was considered to be α AAV-Ab-positive.

The ELISA analyses showed a differential prevalence of α AAV-Abs in MPS III patients compared to healthy controls (Table 2 and Fig. 1a–c). Overall, among all study subjects (aged 2–18 years), the seropositive rates for AAV1–9 ranged from 13% to 33% in

MPS IIIA, from 7% to 14% in MPS IIIB, and from 11% to 37% in healthy controls. AAVrh74 was exceptional, with a much higher Ab incidence of 51–75% among the different study groups (Table 2 and Fig. 1a). A higher incidence of α AAV1-IgG was observed in MPS IIIA patients than in MPS IIIB and healthy control subjects, and a lower IgG incidence for AAV2, 4, 5, 8, and 9 was observed in MPS IIIB patients compared to control and MPS IIIA subjects (Figs. 1a and 2a–c). The combined MPS III subjects showed higher α AAVrh74-Ab incidence and a lower seropositive rate for AAV6 and AAV7 Abs than controls did (Figs. 1a and 2). No differences in α AAV3-IgG incidence were seen among MPS III and control subjects (Figs. 1a and 2). Notably, the majority of the detected differences were statistically insignificant, except that of α AAV6 between MPS IIIA and controls ($p < 0.05$), with close to significance lower incidence of α AAV9-IgG in MPS IIIB ($p = 0.07$) and higher α AAVrh74-Abs in MPS IIIA ($p = 0.07$) than in controls (Fig. 1a and Table 2).

In this study, 3/24 (12.5%) of MPS IIIA patients, 3/14 (21.4%) of MPS IIIB patients, and 8/35 (22.9%) of healthy control subjects were negative for IgG to any of the tested AAV serotypes (Supplementary Table S1), and 14 subjects were seropositive for AAVrh74 only, including 5/12 MPS IIIA, 4/14 MPS IIIB, and 5/35 control subjects ($p > 0.29$; Supplementary Table S1). Further, 36/73 study subjects were seropositive for five or fewer of the tested AAV serotypes, with predominantly low IgG levels ($< 1:200$), including 13/24 MPS IIIA, 7/14 MPS IIIB, and 16/35 controls (Supplementary Table S1). While the α AAV-IgG levels were low in the majority of the seropositive subjects, 13 individuals (3 MPS IIIA, 1 MPS IIIB, and 9 controls) were seropositive for 6–9 of the 10 tested AAV serotypes with

Table 2. Comparison of serum anti-AAV-IgG incidence in MPS III patients and healthy control subjects

AAV serotypes	Number of subjects: anti-AAV-IgG-positive (%)								
	MPS IIIA			MPS IIIB			Controls		
	Total ($n = 24$)	2–7 years ($n = 16$)	>8 years ($n = 8$)	Total ($n = 14$)	2–7 years ($n = 5$)	>8 years ($n = 9$)	Total ($n = 35$)	2–7 years ($n = 18$)	>8 years ($n = 17$)
AAV1	6 (25%)	5 (31%)*	1 (13%)	2 (14%)	2 (40%)*	0****	4 (11%)	1 (6%)	3 (18%)
AAV2	8 (33%)	7 (44%)*	1 (13%)*	2 (14%)	1 (20%)	1 (11%)*	11 (31%)	3 (17%)	8 (47%)*
AAV3	7 (29%)	5 (31%)	2 (25%)	5 (38%)	2 (40%)	3 (33%)	13 (37%)	4 (22%)	9 (53%)*
AAV4	5 (21%)	4 (25%)	1 (13%)	2 (14%)	1 (20%)	1 (11%)	8 (23%)	4 (22%)	4 (24%)
AAV5	3 (13%)	2 (13%)	1 (13%)	1 (7%)	1 (20%)	0**	6 (17%)	1 (6%)	5 (29%)*
AAV6	3 (13%)*	2 (13%)	1 (13%)*	2 (14%)	1 (20%)	1 (11%)*	12 (34%)	3 (17%)	9 (53%)*
AAV7	4 (17%)	3 (19%)	1 (13%)*	2 (14%)	1 (20%)	1 (11%)*	12 (34%)	2 (11%)	10 (59%)*
AAV8	7 (29%)	4 (25%)	3 (38%)	2 (14%)	1 (20%)	1 (11%)*	11 (31%)	3 (17%)	8 (47%)*
AAV9	7 (29%)	3 (19%)	4 (50%)*	1 (7%)*	1 (20%)	0****	11 (31%)	1 (6%)	10 (59%)*
AAVrh74	18 (75%)*	12 (75%)*	6 (75%)	9 (64%)	4 (80%)*	5 (56%)	18 (51%)	6 (33%)	12 (71%)*

Serum samples from MPS III patients and healthy controls were assayed by binding ELISA for total IgG against AAV1–9 and rh74. α AAV-IgG is expressed as ELISA titer, and ELISA titer $\geq 1:50$ is considered seropositive.

* $p \leq 0.05$ vs. controls; ** $p = 0.07$ vs. controls; *** $p = 0.09$ vs. controls; **** $p \leq 0.05$ vs. MPS IIIA; ***** $p \leq 0.05$ vs. 2–7 years; ***** $p = 0.07$ vs. 2–7 years.

AAV, adeno-associated virus; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay.

Figure 1. Prevalence of antibodies (Abs) against different adeno-associated virus (AAV) serotypes in mucopolysaccharidosis (MPS) III patients and healthy children. Serum samples from patients with MPS IIIA (aged 2–18 years; $n=24$) or MPS IIIB (aged 2–20 years; $n=14$) and healthy individuals (aged 2–15 years; $n=35$) were assayed by binding enzyme-linked immunosorbent assay (ELISA) for total immunoglobulin G (IgG) against AAV1–9 and rh74. Serum total α AAV-IgG levels are expressed as ELISA titer, and ELISA titer $\geq 1:50$ was considered as seropositive. (a) all subjects; (b) aged 2–7 years; (c) aged >8 years. * $p < 0.05$ vs. HC; * $p = 0.06$ – 0.07 vs. HC; $^{\dagger}p = 0.09$ vs. HC; $^{\wedge}p < 0.05$ vs. MPS IIIA. HC, healthy controls.

each α AAV-IgG at much higher levels ($>1:400$; Supplementary Table S1).

Although the combined data from all subjects showed no statistically significant differences in overall AAV seropositivity among the three study

groups ($p > 0.2$), further analyses showed age and disease effects in specific α AAV-Ab prevalence, with the highest toward AAVrh74 among all three study groups (Fig. 1b and c and Table 2). Among 2- to 7-year-old subjects, while trends toward higher

Figure 2. Age impacts on prevalence of Abs against different AAV serotypes in MPS III patients and healthy children. Serum samples from patients with MPS IIIA (aged 2–18 years; $n=24$) or MPS IIIB (aged 2–20 years; $n=14$) and healthy individuals (aged 2–15 years; $n=35$) were assayed by binding ELISA for total IgG against AAV1–9 and rh74. Serum total α AAV-IgG levels are expressed as ELISA titer, ELISA titer $\geq 1:50$ was considered as seropositive. (a) healthy controls; (b) MPS IIIA; (c) MPS IIIB. # $p < 0.05$ vs. 2–7 years of age; @ $p = 0.07$ vs. 2–7 years of age.

Ab incidence were observed against the majority of the tested AAV serotypes in MPS III subjects, only α AAV1-IgG and α AAVrh74-IgG incidences were significantly higher compared to controls (Fig. 1b and Table 2). Conversely, in ≥ 8 -year-old subjects, the incidence of α AAV-Abs to virtually all tested AAV serotypes was higher in controls than in MPS III subjects, with the exception α AAVrh74 (Fig. 1c and Table 2). This differential in age of seroconversion between MPS and healthy controls is more easily visualized when the data are plotted by age group for each serotype (Fig. 2). The charts reveal significantly greater increases in the prevalence of Abs against AAV2, 3, and 5–9 and rh74 ($p \leq 0.05$; but no change in α AAV4-Abs) in older healthy controls (aged ≥ 8 years) than in younger (aged 2–7 years) ones (Fig. 2a and Table 2). In contrast, ≥ 8 -year-old MPS IIIA subjects showed lower incidences of Abs to AAV1, 2, 4, and 7, a higher incidence of IgG to AAV8 and 9, and no difference in Abs to AAV3, 5, and 6 and rh74 when compared to the 2- to 7-year-old subjects (Fig. 2b and Table 2), though none of the changes were statistically significant. While the incidence of IgG to all 10 tested AAV serotypes appeared to be reduced, a significant decline ($p < 0.05$) was seen only in α AAV1-Abs, when comparing ≥ 8 -year-old to 2- to 7-year-old MPS IIIB subjects (Fig. 2c and Table 2).

In addition to comparisons of α AAV-Ab prevalence among the different groups, α AAV-Ab titers were also compared to determine whether there were correlations among different AAV serotypes (Fig. 3, Table 3, and Supplementary Table S1). When comparing data from all subjects, in general, no significant differences were observed in the Ab levels against the majority of tested AAV serotypes, with the exception that α AAV3-IgG was significantly higher in healthy controls than in MPS IIIA subjects ($p < 0.05$), or higher, but not reaching statistical significance, than in MPS IIIB subjects ($p = 0.09$; Fig. 3Aa). There were no significant differences in IgG levels to any of the tested AAV serotypes among 2- to 7-year-old MPS III and controls (Fig. 3Ab). However, anti-AAV3-Ab levels were significantly higher ($p < 0.05$) in older healthy controls (aged ≥ 8 years) compared to those in both MPS IIIA and MPS IIIB patients (Fig. 3Ab). Notably, in the older (aged ≥ 8 years) MPS IIIB cohort, there was no detectable IgG against AAV1 or 5–9 or rh74, and low levels of Abs to AAV2 or 4 in only one subject (Fig. 3Ab and B). Further, when looking at the age effects within each cohort, the only significant finding was higher α AAV3-IgG levels in ≥ 8 -year-old healthy subjects compared to the younger controls (Supplementary Fig. S1).

The study also assessed the co-prevalence and correlation of titers of α AAV-Abs between any two serotypes. As shown in Table 4, the co-prevalence was generally not reciprocal between any two specific α AAV-Ab-positive groups due to the large differences in group sizes. The 10 subjects who were positive for α AAV5-IgG had the highest co-prevalence of IgG to the majority of tested serotypes, while the 45 subjects who were positive for α AAVrh74-IgG had the lowest (Table 4). Of α AAV5-Ab-positive subjects, 100% were also positive for α AAV3 and 6 and rh74, 90% positive for α AAV2, 7, and 9, and 80% positive for α AAV1 and 8, with 60% positive for α AAV6 (Table 4). In contrast, among α AAVrh74-positive individuals, only 22–24% were also positive for α AAV1, 4, and 5, 31% positive for α AAV6, 36–38% positive for α AAV7, 8, and 9, 40% positive for α AAV2, and 47% positive for α AAV3 (Table 4). The highest co-prevalence for virtually all tested serotypes was with α AAVrh74-Abs (Table 4), which were detected in 100% of subjects positive for α AAV1 and α AAV5, 84–89% of individuals positive for α AAV2, α AAV7, and α AAV9, and 77–80% of subjects positive for α AAV3, α AAV4, α AAV6, and α AAV8 (Table 4).

The levels of α AAV-IgG from all α AAV-Ab-positive individuals were further analyzed by correlation coefficient in order to assess the potential association or cross-reactivity between different AAV serotypes (Table 5 and Supplementary Fig. S2). The results showed broad correlations in IgG levels to most different AAV serotypes, with the exception of subjects positive for α AAV4 and α AAV5, which showed no significant correlations with those of the other nine tested serotypes (Table 5 and Supplementary Fig. S2). In α AAV1-positive individuals, the IgG levels were significantly correlated with those of α AAV2, 3, and 6–9 and rh74, but not AAV4 and 5 (Table 5 and Supplementary Fig. S2). Similar correlation profiles were observed in IgG levels in subjects positive for α AAV2, 3, or 7, with each showing significant correlation to the majority of tested serotypes, except α AAV4 and α AAV8. Notably, IgG levels in α AAV8- and α AAV9-positive subjects showed less correlation with those of other serotypes, except α AAV1 and α AAV9 for α AAV8-positive subjects, and α AAV1, 2, and 8 and rh74 for α AAV9-positive subjects. In contrast, the Ab levels in α AAV6- or α AAVrh74-positive individuals were significantly correlated with Abs to all eight other serotypes, except α AAV4.

DISCUSSION

This study demonstrates the complex humoral immunity profiles to AAVs among 2- to 18-year-old

A **B**

Figure 3. Levels of IgG against different AAV serotypes in MPS III patients and healthy children. Analyses were performed to determine the variation of α AAV-Ab levels in seropositive MPS III patients and healthy children (**A**, **B**). Serum total α AAV-IgG is expressed as ELISA titer, and ELISA titer $\geq 1:50$ is considered as seropositive. * $p < 0.05$ vs. HC; $^{\dagger}p = 0.06-0.07$ vs. HC; $^{\ddagger}p = 0.09$ vs. HC; $^{\Delta}p < 0.05$ vs. MPS III; 1, $n = 1$; 0, $n = 0$.

healthy individuals and patients with MPS IIIA or IIIB, which suggest broad Ab cross-reactivity across different serotypes. The data also suggest the association of α AAV-Ab prevalence with disease conditions and age. While overall α AAV seropositive prevalence rates appear to be similar among MPS III patients and healthy control children, before 8 years of age, α AAV-IgGs for the majority of serotypes are more prevalent in MPS

III patients than in healthy controls, especially α AAV1 and α AAVrh74, suggesting that the disease pathologies or patient environment may increase exposure or trigger susceptibility to AAV infections. Notably, disease effects on α AAV-Ab prevalence were also reported in patients with Duchenne muscular dystrophy, Becker muscular dystrophy, inclusion body myositis, and GNE myopathy.²¹ Although it is unclear as to the mechanisms in-

Table 3. Levels of anti-AAV-IgG in seropositive MPS III patients and healthy controls

AAV serotypes	Number of subjects: anti-AAV-IgG-positive (ELISA titer)*					
	MPS IIIA			MPS IIIB		
	Age 2-7 years (n = 16)	Age 8-18 years (n = 8)	Age 2-7 years (n = 5)	Age 8-20 years (n = 9)	Age 2-7 years (n = 18)	Age 8-15 years (n = 7)
AAV1	5 (1:50-800, 1:230 ± 325)	1 (1:400)	2 (1:50-1,600)	0	1 (1:800)	3 (1:200-6,400, 1:2,333 ± 3,523)
AAV2	7 (1:50-3,200, 1:183 ± 1,140)	1 (1:1,600)	1 (1:6,400)	1 (1:400)	3 (1:100-6,400, 1:2,200 ± 3,637)	8 (1:200-6,400, 1:1,950 ± 2,171)
AAV3	5 (1:50-800, 1:220 ± 325)	2 (1:50-800)	2 (1:50-1,600)	3 (1:50-200, 1:100 ± 87)	4 (1:50)	9 (1:200-6,400, 2,377 ± 1,893)
AAV4	4 (1:50-800, 1:325 ± 357)	1 (1:100)	1 (1:50)	2 (1:50-100)	2 (1:50-400, 1:138 ± 175)	4 (1:50-200, 1:88 ± 75)
AAV5	2 (1:400)	2 (1:50-400)	1 (1:400)	0	1 (1:200)	5 (1:50-400, 1:230 ± 164)
AAV6	2 (1:200-1,600)	2 (1:50-800)	1 (1:1,600)	1 (1:50)	3 (1:50-800, 1:300 ± 433)	9 (1:50-3,200, 1:700 ± 1,050)
AAV7	3 (1:400-800, 1:533 ± 231)	2 (1:50-400)	1 (1:1,600)	1 (1:100)	2 (1:50-800)	10 (1:50-3,200, 1:545 ± 962)
AAV8	4 (1:50-400, 1:225 ± 202)	3 (1:50-400, 1:183 ± 189)	1 (1:400)	1 (1:50)	3 (1:50-100, 1:67 ± 29)	8 (1:100-6,400, 1:988 ± 2,189)
AAV9	3 (1:100-800, 1:333 ± 404)	4 (1:50-6,400, 1:1,737 ± 3,112)	1 (1:800)	0	1 (1:1,600)	10 (1:50-3,200, 1:475 ± 966)
AAVrh74	12 (1:50-6,400, 1:630 ± 1,817)	6 (1:50-200, 1:127 ± 85)	4 (1:50-400, 1:155 ± 188)	5 (1:50-128, 1:101 ± 32)	6 (1:50-800, 1:233 ± 311)	12 (1:50-16,000, 1:2,504 ± 4,875)

Serum samples were assayed for total IgG against AAV1-9 and AAVrh74 by binding ELISA. Serum IgG is expressed as ELISA titer, and ELISA titer $\geq 1:50$ is considered seropositive. Data presented: number of Ab-positive subjects (IgG titer range, means $\pm$ standard deviation).

Table 4. Co-prevalence of antibodies against different AAV serotypes

IgG	α AAV1 ⁺ (n = 12)	α AAV2 ⁺ (n = 21)	α AAV3 ⁺ (n = 25)	α AAV4 ⁺ (n = 15)	α AAV5 ⁺ (n = 10)	α AAV6 ⁺ (n = 18)	α AAV7 ⁺ (n = 18)	α AAV8 ⁺ (n = 22)	α AAV9 ⁺ (n = 19)	α rh74 ⁺ (n = 45)
α AAV1 ⁺		48% 2 ⁺	36% 3 ⁺	33% 4 ⁺	80% 5 ⁺	44% 6 ⁺	39% 7 ⁺	32% 8 ⁺	37% 9 ⁺	24% 74 ⁺
α AAV2 ⁺	83% 1 ⁺		68% 3 ⁺	53% 4 ⁺	90% 5 ⁺	72% 6 ⁺	78% 7 ⁺	55% 8 ⁺	68% 9 ⁺	40% 74 ⁺
α AAV3 ⁺	67% 1 ⁺	81% 2 ⁺		87% 4 ⁺	100% 5 ⁺	83% 6 ⁺	78% 7 ⁺	55% 8 ⁺	63% 9 ⁺	47% 74 ⁺
α AAV4 ⁺	42% 1 ⁺	43% 2 ⁺	52% 3 ⁺		60% 5 ⁺	50% 6 ⁺	50% 7 ⁺	36% 8 ⁺	26% 9 ⁺	24% 74 ⁺
α AAV5 ⁺	50% 1 ⁺	43% 2 ⁺	32% 3 ⁺	40% 4 ⁺		56% 6 ⁺	50% 7 ⁺	36% 8 ⁺	47% 9 ⁺	22% 74 ⁺
α AAV6 ⁺	67% 1 ⁺	62% 2 ⁺	60% 3 ⁺	60% 4 ⁺	100% 5 ⁺		83% 7 ⁺	55% 8 ⁺	68% 9 ⁺	31% 74 ⁺
α AAV7 ⁺	67% 1 ⁺	67% 2 ⁺	56% 3 ⁺	60% 4 ⁺	90% 5 ⁺	83% 6 ⁺		68% 8 ⁺	68% 9 ⁺	36% 74 ⁺
α AAV8 ⁺	58% 1 ⁺	57% 2 ⁺	48% 3 ⁺	53% 4 ⁺	80% 5 ⁺	66% 6 ⁺	83% 7 ⁺	50% 8 ⁺	57% 9 ⁺	38% 74 ⁺
α AAV9 ⁺	58% 1 ⁺	62% 2 ⁺	48% 3 ⁺	33% 4 ⁺	90% 5 ⁺	72% 6 ⁺	72% 7 ⁺	50% 8 ⁺		36% 74 ⁺
α rh74 ⁺	100% 1 ⁺	86% 2 ⁺	80% 3 ⁺	80% 4 ⁺	100% 5 ⁺	78% 6 ⁺	89% 7 ⁺	77% 8 ⁺	84% 9 ⁺	

Serum IgG is expressed as ELISA titer, and ELISA titer $\geq 1:50$ is considered seropositive.

+, co-prevalence was determined based on data from all individuals who were IgG-positive against each AAV serotype (n).

Table 5. Serotype correlation of α AAV-IgG levels

IgG	α AAV1* (n = 12)	α AAV2* (n = 21)	α AAV3* (n = 25)	α AAV4* (n = 15)	α AAV5* (n = 10)	α AAV6* (n = 18)	α AAV7* (n = 18)	α AAV8* (n = 22)	α AAV9* (n = 19)	α rh74* (n = 45)
α AAV1		$r = 0.681, p < 0.001$	$r = 0.871, p < 0.001$	$r = 0.058, p = 0.845$	$r = 0.314, p = 0.411$	$r = 0.924, p < 0.001$	$r = 0.968, p < 0.001$	$r = 0.449, p < 0.036$	$r = 0.933, p < 0.001$	$r = 0.918, p < 0.001$
α AAV2	$r = 0.663, p = 0.019$		$r = 0.762, p < 0.001$	$r = -0.03, p = 0.921$	$r = 0.079, p = 0.839$	$r = 0.769, p < 0.001$	$r = 0.764, p < 0.001$	$r = 0.244, p = 0.274$	$r = 0.761, p < 0.001$	$r = 0.621, p < 0.001$
α AAV3	$r = 0.8619, p < 0.001$	$r = 0.528, p = 0.014$		$r = -0.04, p = 0.901$	$r = -0.06, p = 0.873$	$r = 0.735, p < 0.001$	$r = 0.771, p < 0.001$	$r = 0.346, p = 0.116$	$r = 0.168, p = 0.491$	$r = 0.842, p < 0.001$
α AAV4	$r = 0.135, p = 0.676$	$r = 0.100, p = 0.666$	$r = -0.02, p = 0.894$		$r = 0.324, p = 0.361$	$r = 0.035, p = 0.890$	$r = -0.68, p = 0.762$	$r = 0.279, p = 0.208$	$r = -0.05, p = 0.837$	$r = 0.143, p = 0.348$
α AAV5	$r = 0.416, p = 0.178$	$r = 0.565, p = 0.008$	$r = 0.586, p < 0.002$	$r = 0.474, p = 0.074$		$r = 0.666, p = 0.003$	$r = 0.606, p = 0.008$	$r = 0.092, p = 0.685$	$r = 0.107, p = 0.667$	$r = 0.509, p < 0.001$
α AAV6	$r = 0.912, p < 0.001$	$r = 0.794, p < 0.001$	$r = 0.887, p < 0.001$	$r = 0.147, p = 0.602$	$r = 0.386, p = 0.271$		$r = 0.936, p < 0.001$	$r = 0.338, p = 0.125$	$r = 0.267, p = 0.269$	$r = 0.882, p < 0.001$
α AAV7	$r = 0.962, p < 0.001$	$r = 0.802, p < 0.001$	$r = 0.873, p < 0.001$	$r = 0.147, p = 0.602$	$r = 0.268, p = 0.486$	$r = 0.947, p < 0.001$		$r = 0.381, p = 0.081$	$r = 0.316, p = 0.187$	$r = 0.836, p < 0.001$
α AAV8	$r = 0.974, p < 0.001$	$r = 0.132, p = 0.568$	$r = 0.364, p < 0.080$	$r = 0.342, p = 0.231$	$r = 0.377, p = 0.283$	$r = 0.872, p < 0.001$	$r = 0.328, p = 0.198$		$r = 0.866, p < 0.001$	$r = 0.454, p = 0.002$
α AAV9	$r = 0.927, p < 0.001$	$r = 0.804, p < 0.001$	$r = 0.845, p < 0.001$	$r = 0.093, p = 0.742$	$r = 0.241, p = 0.503$	$r = 0.834, p < 0.001$	$r = 0.920, p < 0.001$	$r = 0.428, p = 0.047$		$r = 0.806, p < 0.001$
α rh74	$r = 0.906, p < 0.001$	$r = 0.559, p = 0.008$	$r = 0.914, p < 0.001$	$r = 0.157, p = 0.577$	$r = 0.112, p = 0.758$	$r = 0.877, p < 0.001$	$r = 0.815, p < 0.001$	$r = 0.415, p = 0.055$	$r = 0.775, p < 0.001$	

Serum IgG is expressed as ELISA titer, and ELISA titer ≥ 150 is considered seropositive. The levels of anti-AAV-Abs were analyzed for correlation between AAV serotypes.

Data presented: r and value. Values shown in bold have no significant correlation ($p > 0.05$).

+, correlation was determined based on data from all individuals who were IgG-positive against each AAV serotype (n).

volved, previous studies reported impacts of mutation and/or treatment types on α AAV-Ab prevalence in patients with methylmalonic academia (MMA).²² The data also indicate that the seroprevalence for the majority of AAV serotypes peaked in MPS III patients before 8 years of age, except for an increase in α AAV8 and α AAV9 in MPS IIIA. In contrast, there are significant increases in α AAV-Ab prevalence across most tested serotypes in 8- to 15-year-old healthy children. The major age for seroconversion for α AAV-Abs in the healthy population appears to be around 8 to 9 years, which is supported by similar findings for seroprevalence for AAV2 and AAV8 in children.^{23,24} The observed lower α AAV-Ab prevalence in MPS III patients versus healthy children after 8 years of age may be attributable to reduced exposure to AAV because of limited social interactions due to severe progressive neurodegeneration.

One of the interesting findings in this study was the high seroprevalence of α AAVrh74, not only healthy children but also in MPS III patients, with 75% MPS IIIA and 80% of MPS IIIB patients positive for α AAVrh74-IgG before 8 years of age. Notably, the high α AAVrh74-Ab prevalence in MPS III patients was persistent. While AAVrh74 has not been as broadly studied as the more commonly used serotypes in gene therapy, it has recently been shown to have the natural ability to cross the BBB and provide effective gene therapy treatment in MPS IIIA in mice.²⁵ The high seroprevalence may hinder the potential of AAVrh74 as a gene therapy vector by systemic delivery for the treatment of MPS III and other neurological disease in general.

In this study, the complex differential co-prevalence of α AAV-Abs in human subjects largely paralleled the previously characterized structure of evolutionary AAV clades, with the more distantly related serotypes less likely to show co-prevalence.²⁶ While 80–100% of α AAV5-Ab positive individuals were also positive for eight other AAV serotypes, only 22–56% of individuals who were Ab-positive for other AAV serotypes were also AAV5 positive, with no correlation between the levels of α AAV5-Abs and those of other AAV serotypes. Similarly, Ab levels to AAV4 were not correlated with Abs against any other AAV serotypes. This is consistent with the distant relationship between AAV4 and AAV5 compared to other AAV serotypes.²⁶ Interestingly, while low co-prevalence (22–40%) of Abs to AAV1–9 was observed in α AAVrh74-Ab-positive individuals, all or nearly all (77–100%) individuals positive for α AAV1–9 were also positive for AAVrh74, suggesting that AAVrh74

is generally cross-reactive to other AAV serotypes but not the reciprocal. Further, the titers of α AAVrh74-Abs positively correlate with those of the majority of AAV serotypes, except AAV4 and 5. In other words, the α AAVrh74-Abs in the majority of α AAVrh74-Ab-positive individuals may be attributable to the cross-reactive Ab generated by infections with other AAV serotypes, rather than to infection with AAVrh74 itself. While no published structural data are currently available comparing AAVrh74 capsid protein with those of other AAV serotypes, an alignment of the capsid sequences with several other AAV serotypes using BLASTx showed levels of homology consistent with the proposed evolutionary clades,²⁶ with higher homology to AAV1–3 and 6–9 and much lower homology to AAV4 and 5 (Supplementary Table S2). A similar relationship is apparent in an alignment of VP1 capsid amino acid sequences using Vector NTI, showing a close relationship between highly conserved and highly variable capsid regions between all of the serotypes (Supplementary Fig. S2). However, it is currently unclear as to how this may affect the Ab cross-reactivity of AAVrh74 compared to other AAV serotypes.

The majority of AAV seropositive individuals (78%) were positive for one to five of the 10 tested serotypes, generally with very low α AAV-IgG levels, mostly at 1:50–100 titer. Notably, while fewer (22%) individuals were seropositive for six or more AAV serotypes, high levels of α AAV-IgGs for multiple serotypes were detected in the majority of these individuals, suggesting possible Ab responses to more recent infections with at least one specific AAV serotype. The highest levels of IgG appear to be for AAV2, AAV3, and AAVrh74, suggesting an initial infection with AAV2 or AAV3, with the high levels of α AAVrh74-IgG in some individuals attributable to the broad cross-reactivity of this serotypes, as discussed above. Based on these data, it is believed that the majority of patients with MPS III, and the pediatric population <8 years of age in general, would be eligible for gene

therapy using AAV1–9 as vector, given the relatively low seropositive prevalence. Despite the high prevalence of α AAVrh74-Abs, there may also be some potential for this capsid as a gene therapy vector, given that the levels are generally low in the majority α AAVrh74-Ab-positive individuals, and may therefore be less challenging to deplete if effective interventions become available.²⁷

In summary, this study has shown the complex Ab-prevalence profiles for different AAV serotypes in MPS III patients and healthy children, suggesting the potential impacts of disease conditions on AAV seroprevalence. The broad co-prevalence observed in this study indicates that pre-existing α AAV-Abs remain a critical challenge for the translation of AAV gene therapy to treat all patients, regardless of the designated vector serotype. The suggested cross-reactivity among most serotypes implies that this may also be true with novel AAV vectors derived from capsid modifications. It is therefore important to develop approaches for effective Ab depletion, in order to treat as many patients as possible, including patients with naturally acquired α AAV-Abs and those who may need AAV vector re-administration after receiving the initial AAV gene transfer.

ACKNOWLEDGMENTS

This study was supported by funds from Ben's Dream—The Sanfilippo Research Foundation, Liv Life Foundation and Reagan's Hope Foundation. H.F., K.M.F., K.L.M., D.M.M., A.S.M., and R.P. were also supported by a translational research grant from NIH/NINDS (U01NS069626). D.M.M. and H.F. were also supported by a grant from NIH/NCI (R01CA172713).

AUTHOR DISCLOSURE

H.F. and D.M.M. are co-inventors of ABO-101 and ABO-102 of Abeona Therapeutics, Inc., and hold stock of the company. None of the other co-authors have any conflicts of interest.

REFERENCES

1. Neufeld EF, Muenzer J. The mucopolysaccharidoses. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic and Molecular Basis of Inherited Disease*, 8th ed. New York: McGraw-Hill, 2001:3421–3452.
2. Fu H, Dirosario J, Killedar S, et al. Correction of neurological disease of mucopolysaccharidosis IIIB in adult mice by rAAV9 trans-blood-brain barrier gene delivery. *Mol Ther* 2011;19:1025–1033.
3. Fu H, Cataldi MP, Ware TA, et al. Functional correction of neurological and somatic disorders at later stages of disease in MPS IIIA mice by systemic scAAV9-hSGSH gene delivery. *Mol Ther Methods Clin Dev* 2016;3:16036.
4. Ruza A, Marco S, Garcia M, et al. Correction of pathological accumulation of glycosaminoglycans in central nervous system and peripheral tissues of MPSIIIA mice through systemic AAV9 gene transfer. *Hum Gene Ther* 2012;23:1237–1246.
5. Haurigot V, Bosch F. Toward a gene therapy for neurological and somatic MPSIIIA. *Rare Dis* 2013;1:e27209.

6. Rivera VM, Gao GP, Grant RL, et al. Long-term pharmacologically regulated expression of erythropoietin in primates following AAV-mediated gene transfer. *Blood* 2005;105:1424–1430.
7. Nathwani AC, Tuddenham EG, Rangarajan S, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *New Engl J Med* 2011; 365:2357–2365.
8. Manno CS, Pierce GF, Arruda VR, et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* 2006;12:342–347.
9. Nathwani AC, Rosales C, McIntosh J, et al. Long-term safety and efficacy following systemic administration of a self-complementary AAV vector encoding human FIX pseudotyped with serotype 5 and 8 capsid proteins. *Mol Ther* 2011;19:876–885.
10. Foust KD, Wang X, McGovern VL, et al. Rescue of the spinal muscular atrophy phenotype in a mouse model by early postnatal delivery of SMN. *Nat Biotechnol* 2010;28:271–274.
11. Foust KD, Nurre E, Montgomery CL, et al. Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nat Biotechnol* 2009;27:59–65.
12. Duque S, Joussemet B, Riviere C, et al. Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons. *Mol Ther* 2009;17:1187–1196.
13. Mussche S, Devreese B, Nagabhushan Kalburgi S, et al. Restoration of cytoskeleton homeostasis after gigaxonin gene transfer for giant axonal neuropathy. *Hum Gene Ther* 2013;24:209–219.
14. Boutin S, Monteilhet V, Veron P, et al. Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Hum Gene Ther* 2010;21:704–712.
15. Calcedo R, Vandenberghe LH, Gao G, et al. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *J Infect Dis* 2009;199:381–390.
16. Scallan CD, Jiang H, Liu T, et al. Human immunoglobulin inhibits liver transduction by AAV vectors at low AAV2 neutralizing titers in SCID mice. *Blood* 2006;107:1810–1817.
17. Calcedo R, Wilson JM. Humoral immune response to AAV. *Front Immunol* 2013;4:341.
18. Wang L, Calcedo R, Bell P, et al. Impact of pre-existing immunity on gene transfer to nonhuman primate liver with adeno-associated virus 8 vectors. *Hum Gene Ther* 2011;22:1389–1401.
19. Truxal KV, Fu H, McCarty DM, et al. A prospective one-year natural history study of mucopolysaccharidosis types IIIA and IIIB: implications for clinical trial design. *Mol Genet Metab* 2016;119: 239–248.
20. Murrey DA, Naughton BJ, Duncan FJ, et al. Feasibility and safety of systemic rAAV9-hNAGLU delivery for treating mucopolysaccharidosis IIIB: toxicology, biodistribution, and immunological assessments in primates. *Hum Gene Ther Clin Dev* 2014;25:72–84.
21. Zygmunt DA, Crowe KE, Flanigan KM, et al. Comparison of serum rAAV serotype-specific antibodies in patients with Duchenne muscular dystrophy, Becker muscular dystrophy, inclusion body myositis, or GNE myopathy. *Hum Gene Ther* 2017;28:737–746.
22. Harrington EA, Sloan JL, Manoli I, et al. Neutralizing antibodies against adeno-associated viral capsids in patients with mutant methylmalonic acidemia. *Hum Gene Ther* 2016;27:345–353.
23. Erles K, Sebkova P, Schlehofer JR. Update on the prevalence of serum antibodies (IgG and IgM) to adeno-associated virus (AAV). *J Med Virol* 1999; 59:406–411.
24. Calcedo R, Morizono H, Wang L, et al. Adeno-associated virus antibody profiles in newborns, children, and adolescents. *Clin Vaccine Immunol* 2011;18:1586–1588.
25. Duncan FJ, Naughton BJ, Zaraspe K, et al. Broad functional correction of molecular impairments by systemic delivery of scAAVrh74-hSGSH gene delivery in MPS IIIA mice. *Mol Ther* 2015;23:638–647.
26. Gao G, Vandenberghe LH, Alvira MR, et al. Clades of adeno-associated viruses are widely disseminated in human tissues. *J Virol* 2004;78:6381–6388.
27. Velazquez VM, Meadows AS, Pineda RJ, et al. Effective depletion of pre-existing anti-AAV antibodies requires broad immune targeting. *Mol Ther Methods Clin Dev* 2017;4:159–168.

Received for publication June 9, 2017;
accepted after revision August 22, 2017.

Published online: September 18, 2017.