Clinical Considerations for Capsid Choice in the Development of Liver-Targeted AAV-Based Gene Transfer

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As gene transfer with adeno-associated virus (AAV) vectors is starting to enter clinical practice, this review examines the impact of vector capsid choice in liver-directed gene transfer for hemophilia. Given that there are multiple clinical trials completed and ongoing in this field, it is important to review the clinical evidence, particularly as a range of AAV-vector serotypes including AAV2, AAV5, AAV8, and AAV10 have been tested. Although there have been a number of successful trials, the development of two investigational AAV vectors for hemophilia B has been discontinued because they did not meet efficacy and/or safety expectations. Whether this difference between success and failure of gene transfer approaches reflects capsid choice, vector design, manufacturing system, or other variables is a question of great interest. Here, we examine the body of evidence across trials to determine the possible influences of serotype choice on key clinical outcomes such as safety, vector clearance, treatment eligibility, occurrence of transaminase elevations, activation of capsid-directed cytotoxic T cell responses, and clinical efficacy. In summary, gene transfer requires a balance between achieving sufficient transgene expression and minimizing destructive immune responses, which may be affected by AAV-vector serotype choice.

Therapies employing gene transfer using adeno-associated virus (AAV) vectors are being fast-tracked for clinical approval for retinal disease, congestive heart failure, hemophilia A and B, X-linked myotubular myopathy, glioblastoma, glioma, and spinal muscular atrophy.[1,2] The focus of this review will be liver-directed AAV gene therapy for hemophilia, in which there are a number of completed or ongoing phase 1 and 2 trials and phase 3 trials that are recruiting.[3] Given that there are multiple clinical trials in this field, it is important to review the clinical evidence, particularly as a range of AAV-vector serotypes including AAV2, AAV5, AAV8, and AAV10 have been tested. In addition, other AAV serotypes such as AAVhu37, a Clade E AAV that is closely related to AAV8,[4] have been examined in non-human primate (NHP) models.[5] Interestingly, the development of two investigational therapies, DTX101 (rAAV10-hFIX) and BAX335 (AAV8-hFIX), were stopped as they failed to meet manufacturer expectations in terms of efficacy and/or safety. Whether these

failures—and the current apparent successes of other programs—reflect capsid choice, vector design, manufacturing system, or other variables is open to question. Although vector design and manufacturing/production systems are beyond the scope of this review, we will examine the impact of capsid choice by exploring AAV serotypes, the basis for serotype distinction, tropism, transduction efficacy, vector shedding, immune responses to AAV, and the impact of pre-existing neutralizing antibodies (NAb) on transduction efficacy to summarize what is known and identify areas that require further investigation.

AAV Capsid Serotypes

The AAV genome includes *rep* and *cap* genes that encode seven proteins.[6] The *rep* gene encodes four non-structural proteins (Rep78, Rep68, Rep 52, and Rep 40), involved with replication, transcriptional control, integration, and encapsidation. The products of the three *cap* genes (Vp1–3) combine as 50 Vp3, five Vp1, and five Vp2 proteins to form the capsid.[6,7] Capsid assembly is assisted by the assembly-activating protein, a non-structural protein encoded within the *cap* gene, which promotes capsid stability and interactions between the capsid proteins.[8] The AAV capsid includes a core eight-stranded β-barrel motif with large loop insertions between the β strands.[9] The common structural features across serotypes are depicted in Figure 1A,[9,10] suggesting that these features may have specific functional activities (e.g., tissue trophism and cellular transduction) although variable regions within these structures between serotypes may confer distinct serotype-specific functional features as vectors for gene transfer and affect immunogenicity.

Currently, 13 AAV serotypes have been identified, which are differentiated based on surface antigen expression and amino acid sequence differences.[7] AAV have been separated into clades A–F, on the basis of shared serologic and functional attributes, as well as two separate clonal isolates (AAV5 and AAV4) that exhibit greater differences
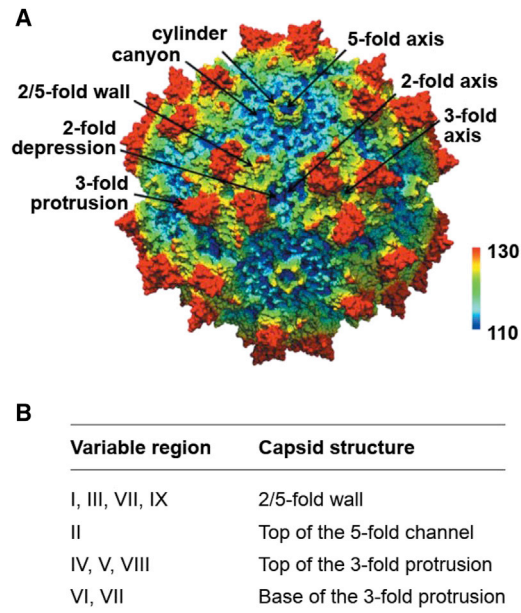
Figure 1. AAV Capsids Share Some Common Structural Features across Serotypes

(A) AAV1 showing common capsid structure features shared with other serotypes. The color coding from blue-green-yellow-red represents the surface topology with the darkest blue representing the lowest areas and the red representing the protruding areas of capsid. (B) Location of the nine variable regions (VRs) in the AAV capsid. Figure reproduced from Tseng and Agbandje-McKenna.[10]

compared with the other serotypes (Figure 2).[7] AAV5 is the most phylogenetically distinct as it shares only 58% capsid homology with AAV2 and AAV8 and 57% homology with AAV10 (Figure 2).[11] In contrast, the other serotypes commonly used in gene transfer share greater homology (e.g., AAV2 shares 83% homology with AAV8 and 84% homology with AAV10).[11] The variance in structure includes conformational differences in regions associated with transduction efficacy and antigenicity, which may be important in terms of differences in tissue tropism, antigenicity, and the likelihood of cross-reactive immunogenicity between serotypes.[7,9,10,12]

Does AAV-Vector Capsid Affect Tissue Tropism?

Tropism can reduce off-target effects by limiting transduction to a particular tissue or cell type and may impact efficacy by concentrating cell transduction in a relevant tissue. Tissue tropism reflects the specific interactions between structures on the AAV-vector capsid that differ between serotypes and glycans (Table 1).[13] The initial binding of many AAV serotypes is via primary receptors including glycans and proteoglycans such as heparan sulfate that are widely expressed in different tissues.[14] This initial binding is followed by interactions with secondary membrane protein receptors that facilitate internalization.[14] A range of secondary receptors has been reported, including the fibroblast growth factor receptor 1 (FGFR1 and alphaV-beta5 integrin) for AAV2,[15] hepatocyte growth factor receptor (c-MET) for AAV2[16] and AAV3,[17] and platelet-derived growth factor receptor

for AAV5.[13] Recently, the protein receptor KIAA0319L (hereafter AAVR) has been identified as critical for the entry of numerous AAV serotypes including AAV1, AAV2, AAV3B, AAV5, AAV6, AAV8, and AAV9.[14] Importantly, the interactions between AAV serotypes and their primary and secondary receptors are likely to affect tissue specificity/tropism (Table 1).[18] In mice, AAV serotypes 1–9 show overlapping but distinct tissue expression patterns: skeletal muscle (AAV 1–9), liver (AAV 1–3 and 5–9), heart (AAV4 and 6–9), lung (AAV4 and 6–9), brain (AAV 8–9), and testes (AAV9).[19] Notably, here are species-specific differences in AAV tropism between mice and nonhuman primates,[20] which raises the question of generalizability of findings across species as well as their relevance for humans.

Given the difficulties in justifying multiple biopsies in the clinical setting, vector biodistribution has not been reported in currently published trials[21–25] and thus data demonstrating tropism profiles in humans is not available. If less invasive, non-biopsy techniques could be developed to assess AAV-vector tropism, this is an area that could be further investigated in future trials. In the absence of such information in humans, tissue-specific promoters are frequently incorporated into vectors to limit the expression of the transgene to a particular target tissue. For example, recent clinical trials employing systemic administration to target the liver utilize vectors with liver-specific promoters: e.g., AAV5-hFIXWT (AMT-060), AAV5-hFIXPadua (AMT-061), SPK-9001, AAV5-hFVIII-SQ (Valoctocogene Roxaparvovec), and scAAV2/8-LP1-hFIXco.[23–26]

Clinical Considerations
AAV-Vector Efficacy

Differences in liver transduction efficacy have been demonstrated in animal models with AAV7 and AAV8-based vectors being approximately 10- to 100-fold more efficient than AAV2- or AAV5-based vectors,[27,28] although AAV tropism in mice is likely to be different from that in NHP and humans. Interestingly, this difference does not appear to reflect the ability of the different serotypes to enter hepatocytes, but instead may reflect more rapid uncoating and conversion of the single-stranded vector DNA into duplex DNA that is transcriptionally active.[27,29] In theory, a more effective vector could be administered at a lower dose compared with a less effective serotype. Administering a lower dose could have potential benefits in terms of reduced immunogenicity. In practical terms, however, a range of factors such as host immunity to the serotype, the quality of vector manufacturing, and transgene activity will also impact the balance between vector dose and the clinical outcomes of gene transfer.

AAV-Vector Clearance

In the field of virology, the term "shedding" is typically used to describe the release of infectious virus from host cells following infection. In the setting of gene therapy, vector shedding is monitored due to the potential risks of vertical transmission to progeny from the presence of AAV vector in semen and from horizontal transmission to close contacts or the wider community via AAV-vector shedding into other body fluids. AAV vectors are designed to be replication
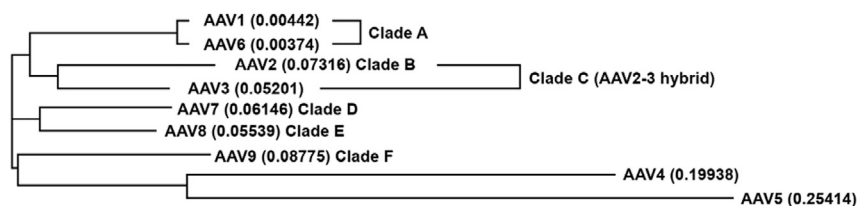
Figure 2. Phylogenetic Relationships among AAV Serotypes

Figure reproduced Drouin and Agbandje-McKenna.[7]

deficient, and thus any vector present would represent material from the primary administration. Monitoring of vector shedding, therefore, represents a measure of clearance of the vector from the body rather than active reproduction of the virus, and, as such, may reflect a number of variables including dose, route of administration, target organ,[30] and potentially interindividual differences. It is important to note that current shedding assays measure vector DNA, so they are unable to distinguish between vector particles versus different forms of DNA (free, episomal, or integrated). Therefore, the detection of vector DNA in body fluids does not necessarily imply an infectious risk. Indeed, in NHP, AAV-vector DNA was detected in all body fluids for up to 6 days after vector transfer, whereas complete AAV-vector particles were only detected in serum for 48–72 h post transfer.[31]

In clinical trials, transient vector shedding was observed for AAV5, AAV2/8, and SPK-9001, although shedding was still detectable in some individuals at week 26 in whole blood for AAV5-hFIXWT 2×10^{13} gc/kg, week 52 in whole blood for AAV5-hFIXWT 5×10^{12}, and week 52 in feces and whole blood for AAV5-hFVIII-SQ (Table 2). Shedding into semen is transient and germ-line transmission has not been observed in animal studies;[30,32] however, physicians should inform individuals that barrier contraception should be practiced as a precaution until clearance of vector DNA in semen is confirmed. It is of interest that vector shedding was more prolonged for AAV5-hFVIII-SQ than AAV5-hFIXWT, which may reflect the

higher doses of AAV5-hFVIII-SQ administered. SPK-9001 and scAAV2/8-LP1-hFIXco were administered at the lowest doses and appeared to have the most transient AAV-vector shedding profile, which also supports a potential dose effect although further investigation is required.

Immune Responses to AAV Vectors

There are two main branches of the adaptive immune system that appear to have the most impact on AAV-vector gene transfer— humoral and cell-mediated immunity. The humoral immune response results in the generation of vector-serotype-specific antibodies, some of which may be neutralizing. Pre-existing neutralizing antibodies to AAV vectors, which may reflect natural exposure to wild-type AAV serotypes or prior AAV-vector exposure, can reduce or prevent successful transduction and thereby impair therapeutic efficacy. Following AAV-vector-based gene transfer, high-titer neutralizing antibodies against the AAV-vector capsid are generated. While these antibodies formed following gene therapy will have no impact on the success of the initial gene transfer, they have implications for readministration of the same serotype and potentially, cross-reactive serotypes. Approaches to circumventing this issue include the use of alternative non-cross-reacting AAV-vector serotypes, which while demonstrated successfully with AAV5 and AAV1 in animal models[33] can be challenging as NAb can cross-react across AAV-vector serotypes, and immunoadsorption/plasmapheresis to reduce levels of circulating NAb.[34]

Cellular immunity includes cytotoxic T cell responses that are usually measured using an enzyme-linked immunosorbent spot (ELISPOT) assay of interferon-γ (IFN-γ) production, which will typically develop 4–12 weeks after gene transfer. One study suggests, however, that memory CD8 cytotoxic T cells in AAV seropositive donor peripheral-blood mononuclear cells (PBMCs) secrete tumor necrosis factor alpha (TNF-α) in response to AAV capsid peptides rather than IFN-γ.[35] Therefore, by focusing on IFN-γ responses, trials may be overlooking key CD8 T cell mediated immune responses to AAV-vector capsids. If confirmed, this may explain some of the discrepancies observed between IFN-γ ELISPOT responses, liver transaminase elevations, and loss of factor activity that are described in the next section.

Murine studies indicate that the development of cytotoxic T cell responses against AAV vectors requires innate immune sensing via Toll-like receptor (TLR) 9 on plasmacytoid dendritic cells.[36] TLR 9 appears to sense the vector genome, as self-complementary AAV2 vectors induced stronger TLR 9 mediated innate responses than single-stranded AAV2 vectors in mice.[37] There is evidence that another TLR (TLR 2) is key for sensing AAV-vector capsid antigens.[38] As we

Table 1. Relationships between AAV Serotype Receptor Usage and Tropism are supported by references[13,14,18,20,59–61]

Glycan Receptor Serotype Usage	AAV Serotype	Impact on Tropism
Heparan sulfate proteoglycan	AAV2, AAV3, AAV6, and AAV13	AAV2 muscle cell interactions
α2–3 and α2–6 N-linked sialic acid (SIA)	AAV1, AAV4, AAV5, and AAV6	AAV5 airway epithelial cell interactions
Laminin receptor	AAV8	widely expressed on tissues targeted by AAV8 including heart, liver, and skeletal muscle
Terminal N-linked galactose of SIA	AAV9	may enable the ability of AAV9 to cross the blood-brain barrier and transduce neural tissues
AAV receptor (AAVR, KIAA0319L)	AAV1, AAV2, AAV3B, AAV5, AAV6, AAV8, and AAV9	AAVR is critical for the entry of numerous AAV serotypes

Table 2. Vector Shedding in Clinical Trials of Liver-Directed Gene Therapy

	SPK-9001 (Serotype Unknown) 5×10^{11} gc/kg[25]	scAAV2/8-LP1-hFIXco 2×10^{12} gc/kg[22]	AAV5-hFIXWT (AMT-060) 5×10^{12} gc/kg[23]	AAV5-hFIXWT (AMT-060) 2×10^{13} gc/kg[23]	AAV5-hFVIII-SQ (Valoctocogene Roxaparvovec) 6×10^{13} gc/kg[24]
Serotype	SPK-100	AAV2/8	AAV5	AAV5	AAV5
Period of Vector Shedding, Weeks (Maximum or Range)					
Nasal secretions	not reported	not reported	18	12	not reported
Saliva	4–6	2	20	16	40–52
Feces	not reported	2	16	20	present to final assessment (week 52)
Urine	2–8	not reported	11	22	6–28
Semen	4–12	2	48	22	36–56
Whole blood	22–42 (PBMCs)	2 (plasma)	present to final assessment (week 52)	present to final assessment (week 26)	present to final assessment (week 52)

Gc, genome copies; PBMCs, peripheral-blood mononuclear cells.

discuss in the next section, there appear to be serotype-specific differences in immune responses to AAV vectors in clinical trials. As more information emerges on the interactions between different AAV serotypes and the innate and adaptive immune systems, the reasons for these differences may become clearer. Given the potential for TLRs to recognize differences in AAV-vector genomes, as well as vector capsid, other factors in addition to AAV serotype, such as the use of self-complementary AAV vectors or codon optimization, may affect immunogenicity.

The Impact of AAV-Vector Serotype on Immune Responses and Liver Toxicity/Loss of Efficacy

AAV-vector-mediated liver toxicity indicated by alanine aminotransferase (ALT) elevations and activation of capsid-specific CD8 T cells has been associated with subsequent decline in FIX and FVIII activity in clinical trials with AAV2, AAV8, and AAV10 (Tables 3 and 4).[39–41] In contrast to these findings, with AAV5-based vectors, there was no observed connection between ALT or aspartate aminotransferase (AST) elevations, cytotoxic T cell responses, and reduction of factor activity (Tables 3 and 4) in clinical trials to date (0/10 participants with AAV5hFIX and 1/8 participants with AAV5hFVIII-SQ).[23,24] The lack of T cell responses and maintenance of factor activity in the presence of transaminitis in both AAV5-vector-based trials in hemophilia[23,24] and similar lack of immune response in the porphyria trial[42] provide initial indications of serotype-specific differences in the generation of capsid-specific T cell responses, although this will need to be confirmed in further studies (Tables 3 and 4). In addition, this evidence, along with findings of inconsistent relationships between transaminase elevation and T cell responses from the scAAV2/8-LP1-hFIXco trial, suggests that ALT and/or AST elevations may not always signal the destruction of transduced hepatocytes.[21,23]

The best way to control immune responses to AAV vectors remains to be determined. Clinically, the use of prophylactic versus on-demand immunosuppression, high-activity transgenes that enable lower doses of vector to be used, and engineering of the capsid surface to reduce immunogenicity have been proposed and are being investigated. In terms of liver toxicity, there is a need to clarify the relative impact

of immune responses, the potential for AAV vectors to induce direct hepatocyte stress responses (whether to AAV-vector capsid or transgene), and the potential for toxicity related to concomitant drug use (e.g., efavirenz for HIV infection).[43] Therefore, it should be considered whether it would be advisable to collect liver tissue biopsies in future trials so that this issue can be clarified.

AAV-Vector Serotypes and Neutralizing Antibodies

Anti-AAV NAb have historically been believed to diminish the efficacy of AAV-based therapies delivered systemically in humans, on the basis of results from preclinical and clinical trials utilizing AAV2 and AAV8. In humans, pre-existing NAb at titers as low as 1:17 for AAV2[44] and 1:1 for the bioengineered capsid AAV-Spark100[25] were associated with reduced, or abrogated, therapeutic efficacy (Table 5). These observations have led to the exclusion of subjects with even low levels of anti-AAV NAb from the majority of AAV-vector-based gene therapy trials up until now. In contrast to other AAV-vector serotypes, successful liver transduction was achieved with AAV5 vector in both NHP and humans with pre-existing anti-AAV5 NAb titers up to 1:1,030 for NHP and 1:340 for humans (Table 6).[23,45,46] Based on data such as this, gene transfer using AAV5 vectors is being investigated in participants with titers of NAbs to the serotype in trials in hemophilia A and B.[47,48]

There is a clear need for a standardized approach for measuring NAbs, so that the cut offs for titers that could cause a clinically relevant impairment of gene transfer can be identified. Importantly, such cut-offs will likely need to be vector serotype and assay specific. As Table 5 indicates, clinical trials include different assays such as inhibition of transduction and direct measurement of antibody titers as well as different cut-offs for demonstrating positivity, so it is impossible to compare findings between studies. An aligned approach to defining clinically relevant titers will be key as gene therapy enters clinical practice.

Due to the high degree of conservation in the amino acid sequence among AAVs,[49] anti-AAV antibodies show cross reactivity with a wide range of serotypes.[50] AAV2 has the highest seroprevalence of NAb in the general population,[50] which may make this serotype most

Table 3. Immunogenicity of Different AAV Gene-Transfer Preparations for Hemophilia B

Parameter	AAVrh10FIX[62,67]	rAAV2hFIX[44]	BAX 335[63,64]	scAAV2/8-LP1-hFIXco[21,22]	SPK-9001 (AAV-FIX)[25]	AAV5hFIX[23]
Serotype	AAV10	AAV2	AAV8	AAV2/8	not reported	AAV5
Dose, gc/kg (n)	Co. 1: 1.6×10^{12} (3) Co. 2: 5.0×10^{12} (3)	2×10^{12} (2)[a]	Co. 1: 2×10^{11} (2) Co. 2: 1×10^{12} (3) Co. 3: 3×10^{12} (2)	Co. 1: 2×10^{11} (2) Co. 2: 6×10^{11} (2) Co. 3: 2×10^{12} (6)	5×10^{11} (10)	Co. 1: 5×10^{12} (5) Co. 2: 5×10^{13} (5)
Transgene	WT	WT	Padua	WT	Padua	WT
Follow up, weeks	10–52	14	7-104	166 (median)	49 (mean)	26–52
Transgene activity, % or IU/dL	5%–20% (peak)	3–11	0.5-≥25	1–6	33.7 (mean)	3–13
ALT elevations	5/6	1/2, same participant experienced AST elevation	2/2 in Co. 3	4/6 in co. 3	2/10, same participants experienced AST elevation	3/10
AST elevations	not reported	1/2, same patient experienced ALT elevation	not reported	1/6, participant with the highest ALT elevation	2/10, same participants experienced ALT elevation	No
Capsid-directed T cell activation	4/6	yes (only reported for 1 participant in the 4×10^{11} gc/kg group)	2/2 in Co.3	yes, Co. 2 and 3	yes (2/2 with ALT elevation)	0/3
Immune response steroid responsive	no	not applicable	no, possibly due to delayed start	yes	yes	Yes
Loss of FIX expression	yes, 5/6	yes, 2/2 in participants with ALT elevations	yes, 2/2 in participants with ALT elevations + T cell response	yes, 4/4 participants with ALT elevations + T cell response	yes, 1/2 with ALT/AST elevations + T cell response	No

ALT, alanine aminotransferase; AST, aspartate aminotransferase; Co., cohort; FIX, factor IX; gc, genome copies; WT, wild type.
[a]Lower doses were tested but did not result in a detectable increase in FIX activity.

suitable for applications for which NAb are less of a concern, such as gene transfer to the eye[51] or brain parenchyma.[52] AAV5, which has the least-conserved capsid sequence versus other serotypes,[53] and the non-human primate serotype AAV8,[49] are among the AAV vectors with the lowest seroprevalence of NAb in humans.[50,54] The seroprevalence of NAb to AAV-vector serotypes also varies by geography, with a higher prevalence of AAV2 NAbs in Africa versus other regions.[54] Therefore, in terms of AAV-vector capsid choice, based on rates of pre-existing immunity it makes sense to choose a vector serotype with the lowest prevalence in the general population such as AAV5 or 8.

There does not appear to be an association between pre-existing NAb and subsequent T cell responses. From the re-analysis of the AAV5-hFIXWT trial samples, the three participants with pre-existing NAb did not experience ALT elevations and did not develop T cell responses.[45] In agreement with these data, in other human and animal trials, pre-existing immunity does not tend to be associated with subsequent T cell responses, and T cell responses may occur in patients without evidence of pre-existing immunity (Table 6). In addition, there does not appear to be a link between the presence of NAbs and subsequent loss of factor activity as this was observed in some trials but not others.

Pre-existing NAb to AAV-vector serotypes are a major limitation in terms of patient access to gene therapy and so far, these patients have been excluded from gene therapy trials.[40] It is possible that sequential administration of non-cross-reacting serotypes may enable re-dosing; as was alluded to earlier in the manuscript, this approach has been successfully demonstrated with AAV5 and AAV1 in NHPs, but has yet to be examined in humans.[33] Also in a NHP model, pre-existing immunity following AAV5-human embryonic alkaline phosphatase (SEAP) treatment was bypassed by immune adsorption allowing successful transduction with AAV5-hFIX.[55] In a mouse model, co-administration of an AAV vector with tolerogenic nanoparticles blocked anti-AAV immune responses and allowed for effective re-dosing.[56] In the future, the use of rational design to alter AAV-vector capsids to avoid pre-existing immunity may be an option.[57] Other potential approaches that need further investigation include better immunosuppression regimens, using the lowest possible AAV-vector dose to achieve efficacy while minimizing immune responses, reducing the total capsid exposure by ridding preparations of empty capsids or conversely using empty capsids as decoys, improving manufacturing quality, and reducing potentially immunostimulatory contaminants.[40] As therapies enter the clinic, studies examining re-dosing will be a key area for further research.

Discussion

The choice of the most appropriate AAV vector for therapeutic gene delivery depends on a number of factors including the prevalence of NAb to the serotype, tissue tropism, and the risk of immunogenicity. The ideal AAV vector, therefore, would have a low seroprevalence and titer of NAb to allow the widest possible patient access to

Table 4. Immunogenicity of Different AAV Gene-Transfer Preparations for Hemophilia A and Porphyria

Parameter	AAV5hFVIII-SQ (Valoctocogene Roxaparvovec)[24]	SPK-8011 AAV-VIII[41]	GO-8 AAV8-HLP-hFVIII-V3 [65]	rAAV2/5-PBGD[42]
Serotype	AAV5	Not reported	AAV8	AAV2/5
Dose, gc/kg (n)	6×10^{12} (1)	5×10^{11} (2)	6×10^{11} (1)	5×10^{11} (2)
	2×10^{13} (1)	1×10^{12} (3)	2×10^{12} (2)	2×10^{12} (2)
	6×10^{13} (6)	2×10^{12} (7)		6×10^{12} (2)
				1.8×10^{13} (2)
Transgene	B-domain–deleted hFVIII	B-domain-deleted hFVIII	17 amino-acid peptide with six N-linked glycosylation motifs from the human FVIII B-domain	WT
Follow up, weeks	52	46	13–47	52
Transgene activity, % or IU/dL	2–164	13-30	7–69	subclinical
ALT elevations	8/9 (low and high)	steroids in 7/12 due to declining FVIII, ALT elevations, or IFN-γ ELISPOT	2/3	1/8 (high)
AST elevations	3/9	not reported	not reported	not reported
Capsid-directed T cell activation	0/9	steroids in 7/12 due to declining FVIII, ALT, or IFN-γ ELISPOT	not reported	0/8
Immune response steroid responsive	no	yes	yes	not reported
Loss of FVIII expression	1/8 with ALT elevations	yes, 7/7	no	not applicable

ALT, alanine aminotransferase; AST, aspartate aminotransferase; Co., cohort; FVIII, factor VIII; gc, genome copies; PBGD, porphobilinogen deaminase; WT, wild type.

treatment, would transfect the tissue of choice, and would not elicit immune responses that impact transgene expression. More needs to be elucidated regarding potential AAV-vector serotype-specific differences in the intracellular processing, and transduction of that may affect clinical outcomes in gene therapy.

For liver-directed gene transfer, a number of AAV-vector serotypes have been trialed including AAV2, AAV5, AAV8, and AAV10. In terms of the capsid structure, AAV5 is the most phylogenetically distinct vector serotype, whereas other commonly used serotypes such AAV2 and AAV8 share over 80% homology. This may impact the prevalence of NAb to AAV5 in the general population, which tend to be lower with AAV5 compared with serotypes such as AAV2 and AAV1. Pre-existing NAb to AAV can impair transduction efficacy and for certain AAV-vector serotypes the prevalence of NAb can reach up to 60%,[50] which could limit patient access to treatment. Pre-existing NAb to AAV5 at titers commonly observed in the general population do not appear to affect transduction efficacy, and some ongoing trials with AAV5-based vectors will include individuals with NAb.[45,47,48,58] Use of diverse AAV-vector serotypes may also permit re-administration in the future due to decreased likelihood of cross-reactive antibodies raised after the first administration. As gene therapy becomes more established, it will be important to standardize NAb assays and define clinically relevant levels to ensure better patient access to gene therapy.

Tropism is key to targeting gene expression to appropriate tissues and to reduce off-target effects. There is increasing evidence elucidating the molecular interactions that underlie tropism, and in animal studies, AAV-vector capsids have been engineered to modify tropism. However, animal models may be poorly predictive of tropism in humans. Additionally, tropism is more difficult to study in humans, so most clinical approaches depend on a tissue-specific promoter to drive expression. This is an area that requires significant further study in humans, although less invasive methods than are currently available are required to enable this.

Vector clearance is relevant due to the risk of horizontal or vertical transmission of infectious AAV vectors, although in reality the infection risk from AAV vectors is low as they are non-pathogenic and replication deficient. Additionally, because current assays assess

Table 5. NAb Exclusion Criterion across Trials of Liver-Directed Gene Transfer for Hemophilia

Developmental Therapeutic	Neutralizing Antibody Definition
AAV5-hFIXWT (AMT-060)	29% inhibition of transduction versus pooled NAb-negative human sera[23]
SPK-9001	AAV-Spark100 neutralizing antibody titer >1:5[25]
scAAV2/8-LP1-hFIXco	no AAV8 NAb based on an in vivo transduction inhibition assay[22]
AAV5-hFVIII-SQ (Valoctocogene Roxaparvovec)	no detectable immunity to AAV5 established with a cell-based transduction inhibition assay and an assay of total AAV5 immunoglobulin[24]
	future studies may focus on total immunoglobulin only as positive results in the transduction inhibition assay did not impact efficacy in a non-human primate study[66]
AAVrh10	> 1.5[67]

Table 6. Pre-existing AAV Immunity and Subsequent T Cell Responses

	AAV5[55]	rAAV2hFIX[44]	AAV5-hFIXWT[23,33]	SPK-9001[25]	scAAV2/8-LP1-hFIXco[21,22]	AAV5-FVIII[24]	AAVrh10[62,67]
Study type	non-human primate	clinical	clinical	clinical	clinical	clinical	clinical
Pre-existing immunity	yes	1/2 (1:2 and 1:17)	3/10	1/10	no	no	yes (NAb titer < 1:5)
T cell response	no	1 (not in the participant with pre-existing immunity)	no	2/10 (not participants with pre-existing immunity)	8/10 (intermediate and high dose group)	no	yes 6/6
Efficacy in participants with pre-existing immunity	no	no	yes	yes, but FIX expression lowest (approx. 10%–15%)	not applicable	not applicable	yes
Loss of FIX expression over time in participants with pre-existing immunity	not applicable	yes, peaked at 2 weeks	no	no	not applicable	not applicable	yes, peaked between 3-14 weeks

NAb, neutralizing antibody.

vector DNA, "shedding" data does not distinguish between vector DNA that is part of an infectious AAV particle and vector DNA fragments that have no infectious risk. Vector shedding was largely transient across clinical trials although in some studies vector shedding was detected in some body fluids up to the last endpoint. There are initial indications that lower-dose AAV vectors may be associated with a shorter duration of vector shedding, but this needs to be confirmed. It is also possible, however, that the duration of shedding is similar but that in some cases the magnitude of vector DNA present is below the limits of detection.

AAV-vector trials have largely demonstrated a modest dose response in terms of transgene expression; however, in some trials, higher doses have been associated with T cell mediated immune responses and associated loss of transgene expression. Therefore, in addition to dose, there may be inherent differences between serotypes in terms of the type of immune responses they elicit and the doses required to do so. For example, with scAAV2/8-LP1-hFIXco, SPK-9001, rAAV2hFIX, and AAVrh10FIX; in some cases, liver damage indicated by ALT elevations is associated with T cell immune responses and subsequent FIX decline. With AAV5-based vectors, in contrast, there did not appear to be an association between ALT elevations, cytotoxic T cell responses, and reduction of FIX activity. Although this provides an initial indication of differences in the immunogenicity of AAV-vector serotypes, our understanding of immune responses to AAV vectors is still at an early stage. Additionally, there is no standardized approach to control immune responses, e.g., by vector dose minimization, vector design, serotype usage, and/or prophylactic or on-demand steroids.

Conclusions

Several factors enter into the consideration of capsid choice for treating patients with gene transfer. A balance must be struck between achieving sufficient transgene expression for clinical benefit and activation of the body's immune expression. Although dose may be a factor, there are initial indications that there may be

inherent differences in immunogenicity between AAV-vector serotypes. Evidence with each serotype is currently limited because only tens of patients have received each construct. As gene transfer becomes more established in the clinic, these gaps in the evidence base should be addressed.

AUTHOR CONTRIBUTIONS

All of the authors contributed to the development of the review from the initial concept stage, provided critical input during the draft stages, and approved the final version prior to submission.

CONFLICTS OF INTEREST

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