Chapter 5

AAV Biology, Infectivity and Therapeutic Use from Bench to Clinic

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Additional information is available at the end of the chapter

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Abstract

Adeno-associated virus (AAV) has been isolated from numerous vertebrate species since 1966. Besides its wide and promiscuous tropism, AAV infection does not result in considerable toxicity or pathogenicity and is capable of achieving adequate and long-term levels of gene transfer, especially following generation of the AAV recombinant variant: rAAV. Due to these properties, rAAV has gained special attention as a viral vector for gene therapy in the last decade. Currently, there are 130 clinical trials taking place worldwide for several diseases testing the safety and efficacy profiles of rAAV. During preclinical and clinical studies, several challenges have arisen in terms of reaching the full therapeutic potential of rAAV, such as efficient delivery of the virus in a targeted and specific manner to a desired tissue. Importantly, the development of immune responses towards the viral capsids poses an obstacle to rAAV applicability in the clinical setting. Numerous approaches have been developed in order to tailor an optimized therapeutic virus for treating specific diseases, including the use of different AAV serotypes or the creation of recombinant capsid variants with distinctive transduction and immunological profiles. This chapter reviews current information on rAAV clinical trials and the potential for combining rAAV platform with other technologies, such as induced pluripotent cells and gene editing.

Keywords: Adeno-associated virus, Gene therapy, Clinical trials

1. Introduction

Gene therapy is currently one of the most promising technologies for the treatment and/or cure of several genetic diseases. Furthermore, it has the potential to battle inherited disorders as

well as acquired diseases. By inducing modification of the gene pool, gene therapy aims to permanently and non-invasively treat the disease. Among the gene modifications that the therapy allows, a gene could be added, by direct introduction of a gene copy, silenced, by administering shRNA or siRNA, or removed, by the ZFN technology. Therefore, the spectrum of diseases that could potentially benefit from this technology is expanding.

Even though the idea of gene transfer has been pursued for decades using an array of diverse delivery approaches, several setbacks hampered its success for some time. In 1999, the death of Jesse Gelsinger after receiving an adenoviral-based gene therapy for the treatment of severe combined immunodeficiency disorder forced the halt on gene therapy progress [1]. Following this tragic incident, a more serious regulatory scrutiny was established and the use of alternative viral and nonviral vectors was investigated. Among viral platforms for gene delivery, adeno-associated virus (AAV) emerged in 1965 and has attracted much attention since then because the virus is not pathogenic, does not induce significant immune response and/or toxicity to humans while it allows long-term transgene expression.

2. Emergence of rAAV as a therapeutic platform

Adeno-associated virus was first discovered in 1965 as a contamination of rhesus monkey kidney cell cultures that were infected with adenovirus stocks [2]. Initially, the virus was called defective as it was incapable of self-replicating in the absence of a helper virus, adenovirus or herpesvirus. Later, it was classified as a member of the Parvovirus family, genus Dependovirus.

Further investigation determined that it is a small virus (approximately 20 nm) composed of an icosahedral protein capsid, which contains single-stranded DNA of 4.7 kb. The viral genome is flanked at each end by inverted terminal repeats sequences of 145 bp called ITRs. These sequences self-assemble into hairpin structures, generating a double-stranded sequence, which serves as a template for replication. The viral genome encodes for two proteins: Rep and Cap. Rep is required for single-stranded DNA replication and packaging. Cap is necessary to form the viral capsid and transduce cells efficiently.

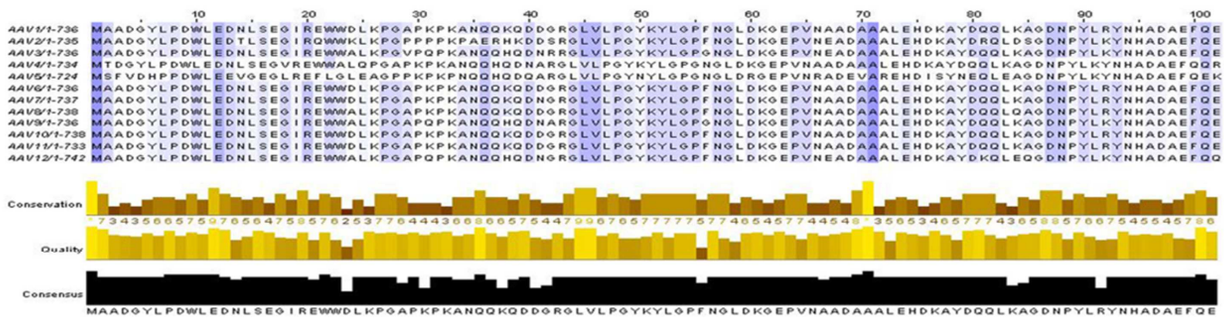
AAV has never been associated with a disease or pathology [3]. Furthermore, due to the homology between the Rep-binding element present on the ITR, and the rAAVS1 sequence found on human chromosome 19, the viral genome can result in integration into the human genome [4]. This last feature is important because it shows that the virus can facilitate long-term expression of the viral genome. Additionally, specific integration of AAV in a defined locus minimizes the risks of mutagenesis due to random insertions, as other vectors do.

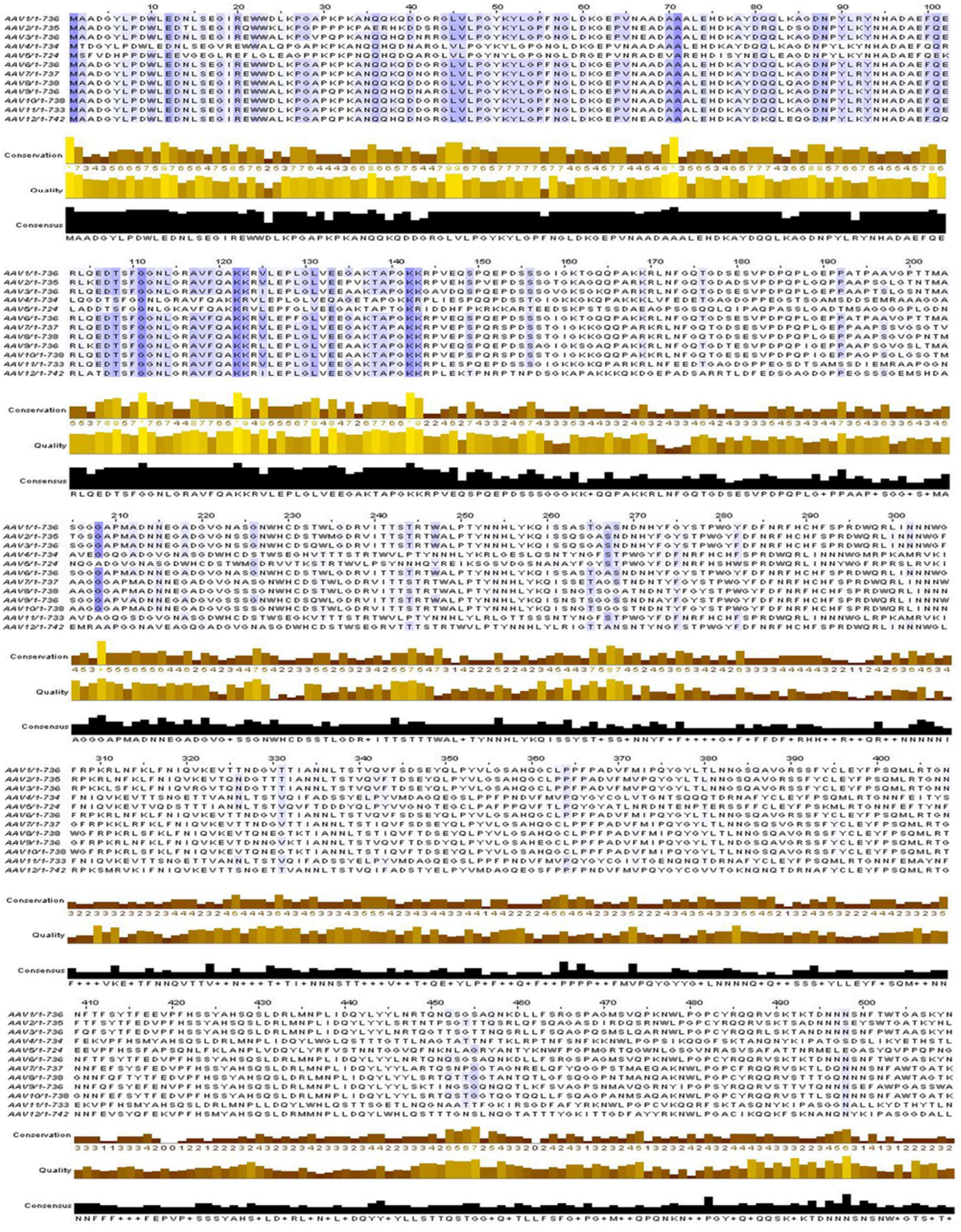
However, several genetic modifications of AAV have been performed in order to guarantee further safety for its translation into the clinic. First, the gene required for viral replication, called Rep, and the element required for site-specific integration were eliminated from the AAV genome. Therefore, this AAV variant, called recombinant vector (rAAV), will exist in an extrachromosomal state with very low integration efficiency into the genomic DNA, reducing

the possibility of inducing random mutagenesis. Second, packaging of the rAAV genomic DNA was modified, incorporating a self-complementary rAAV genome rather than a single-stranded DNA genome [5]. Self-complementary virus differentiates from the recombinant virus in its ability to refold into double-stranded DNA, bypassing the synthesis of the second strand. This substitution has the advantage of reducing the lag time prior to transgene expression and consequently, increasing the biological efficiency of gene delivery. However, it significantly reduces the size of the transgene that could be inserted into the rAAV genome, from 5 kb to 3 kb. Third, several capsid serotypes that carry the rAAV genome have been identified and isolated.

3. AAV capsid serotypes

Even though serotype 2 has been more extensively used and studied, other capsids are gaining more interest. The existence of a variety of serotypes makes rAAV gene therapy more attractive as they differ in infectivity rates and tissue specificity. For instance, a biodistribution analysis of different AAV capsid serotypes carrying the same luciferase reporter gene showed a broad dissemination of the virus in the mouse following intravenous administration [6]. In an attempt to study phylogenetic relationships among serotypes 1 to 12, their capsid amino acid sequences (NCBI reference sequences: NP_049542.1, YP_680426.1, NP_043941.1, NP_044927.1, YP_068409.1, AAB95450.1, YP_077178.1, YP_077180.1, AAS99264.1, AY631965.1, AY631966.1 and AX753364.1) were aligned using ClustalOmega [7] and JalView, version 2.8.2 (Figure 1). According to the degree of similarity that a residue has with the consensus residue for each column, a certain color is given. Intensive blue corresponds to more than 80% agreement, light blue to agreement between 60% and 80%, light grey to agreement between 40% and 60% and white for agreement lower or equal to 40%. Below the alignment, conservation, quality and consensus information are provided. Conservation graphic highlights alignment regions where physicochemical properties are conserved. The more intense the color, the more conserved the physicochemical property is in the column. Alignment quality indicates the likelihood of observing substitutions in a particular amino acidic position. Finally, the residue consensus provides the most common residues and their percentage for each column of the alignment.

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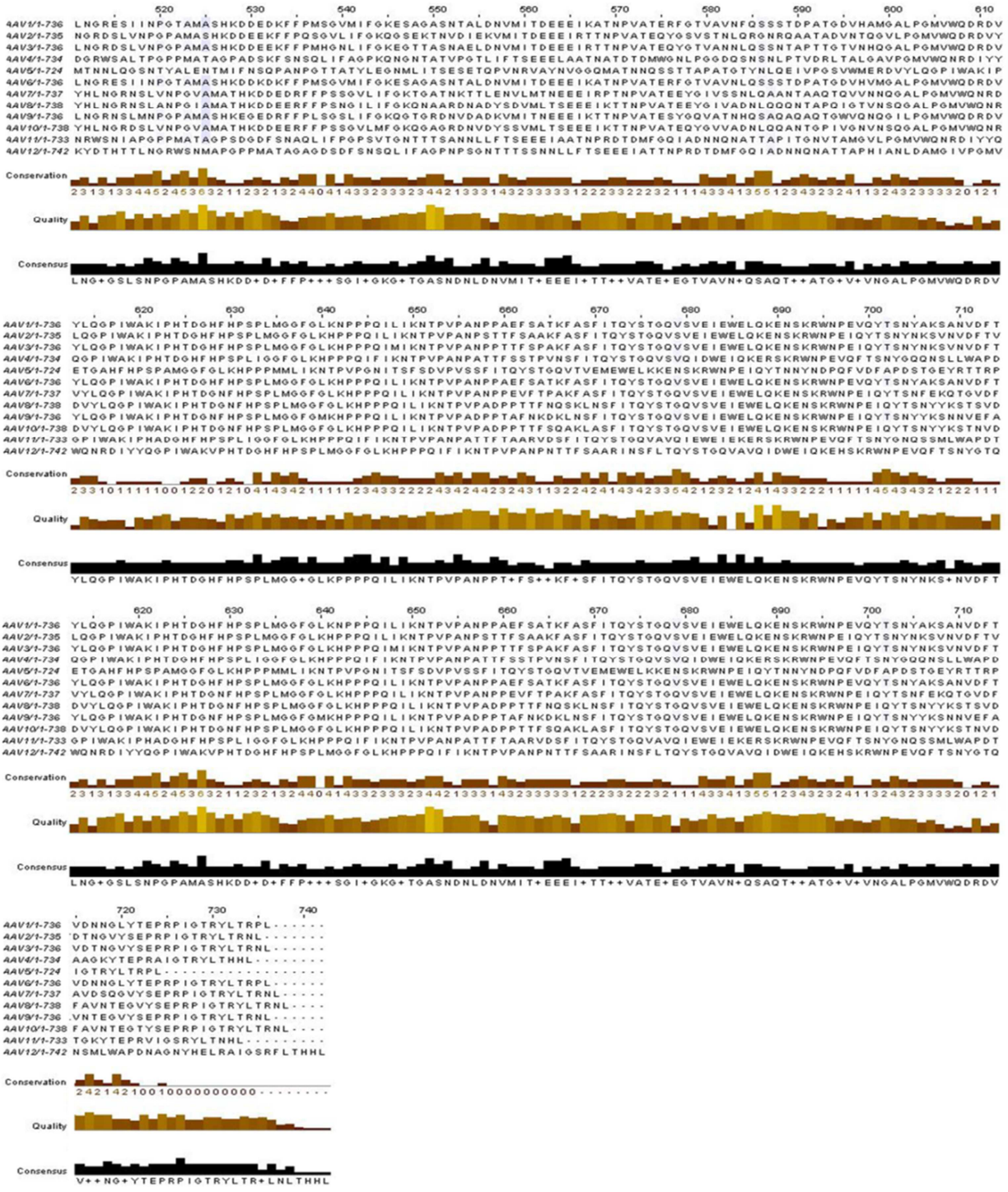

Figure 1. Multi-sequence alignment of AAV serotypes from 1-12 using ClustalOmega and JalView software.

Following the multisequence alignment, the percentage of sequence homology was determined by performing BLAST alignments of dual AAV sequences at the time (Figure 2a). Furthermore, the phylogenetic relationships among these AAV serotypes were determined by creating a neighbor-joining tree (Figure 2b), which uses the percent identity [8].

A

	AAV1	AAV2	AAV3	AAV4	AAV5	AAV6	AAV7	AAV8	AAV9	AAV10	AAV11	AAV12
AAV1	100%	83%	86%	64%	59%	**99%**	85%	84%	82%	85%	67%	61%
AAV2	83%	100%	87%	61%	58%	83%	82%	83%	82%	84%	63%	60%
AAV3	86%	87%	100%	63%	58%	87%	84%	85%	83%	85%	65%	61%
AAV4	64%	61%	63%	100%	53%	63%	64%	64%	63%	64%	82%	79%
AAV5	59%	58%	58%	53%	100%	59%	59%	58%	57%	57%	53%	53%
AAV6	**99%**	83%	87%	63%	59%	100%	85%	84%	82%	85%	66%	61%
AAV7	85%	82%	84%	64%	59%	85%	100%	88%	81%	88%	67%	62%
AAV8	84%	83%	85%	64%	58%	84%	88%	100%	85%	**93%**	66%	62%
AAV9	82%	82%	83%	63%	57%	82%	81%	85%	100%	86%	64%	60%
AAV10	85%	84%	85%	64%	57%	85%	88%	**93%**	86%	100%	67%	61%
AAV11	67%	63%	65%	82%	53%	66%	67%	66%	64%	67%	100%	84%
AAV12	61%	60%	61%	79%	53%	61%	62%	62%	60%	61%	84%	100%

B

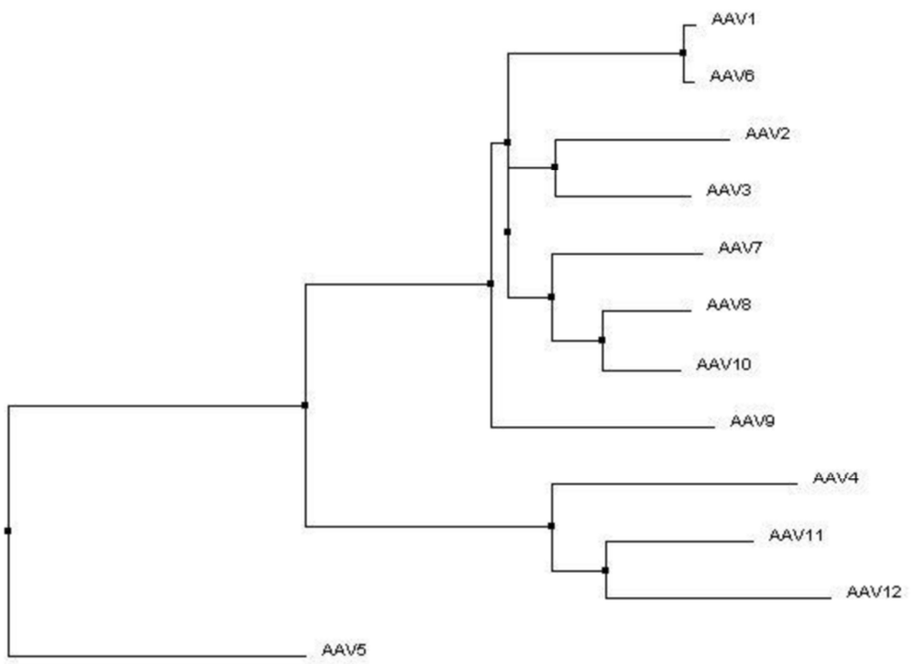

Figure 2. A, Blast alignment of dual combination of AAV serotypes to determine percentage of homology. **B**, Phylogenetic tree to determine phylogenetic relationships among the studied serotypes.

This tree shows that serotype AAV5 has the most divergent amino acid capsid sequence, sharing between 53% and 59% homology with the rest of the human serotypes that have been discovered so far (highlighted in orange). AAV4 also shows a considerable degree of divergence, when comparing sequences of AAV1 to 9 (between 53% and 64%). However, AAV4 shares a more recent common ancestor with serotypes 11 and 12. Furthermore, AAV1 and AAV6 share 99% homology, being the closest AAV serotypes in sequence. The most common AAV serotype, AAV2, is closer in amino acid sequence to all the AAV serotypes, especially AAV3, but greatly differs from serotypes AAV5, AAV4 and therefore, AAV11 and AAV12. Serotypes AAV8 and AAV10 are also very close between each other, sharing 93% amino acid sequence homology. These differences in sequences were observed in other studies, not only when analyzing the sequence similarities but also when studying antigenic reactivities [9]. Remarkably, the variabilities in amino acidic sequences were mainly localized in the looped-

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out domains that are exposed to the surface of the capsid, rather than evenly distributed along the capsid protein sequence [10]. More interesting, Gao et al. compared phylogenies from human and nonhuman primate AAV serotypes. They observed that human AAV4 and AAV5 serotypes were the most divergent, and after they emerged, the rest of the viruses were clustered in groups that included human serotypes (AAV1, AAV6, AAV2, AAV3 and AAV9), exclusive rhesus serotypes (AAV7) or a combination of both (AAV8). Considering that human AAV serotypes share a high similarity in sequences with nonhuman AAV serotypes, they are both well disseminated and are able to cross species barriers. Therefore, there is a possibility that AAV from nonhuman primates could be used for treating human diseases. This is the case of AAVrh10, a serotype isolated from rhesus macaques. This virus was found to be significantly more efficient in transducing neurons from different areas in a healthy dog brain as compared with AAV1 or AAV5, but to a similar extent with AAV9 [11]. More importantly, the rhesus serotype is currently being tested for safety and efficacy in the clinic for the treatment of CNS diseases, such as Battens (NCT01414985 and NCT01161576, clinicaltrials.gov) and MLD (NCT01801709, clinicaltrials.gov). Additionally, a new study is planning to test the safety of this virus for delivering human alpha 1-antitrypsin cDNA to individuals with alpha 1-antitrypsin deficiency (NCT02168686, clinicaltrials.gov); although they are not yet recruiting patients.

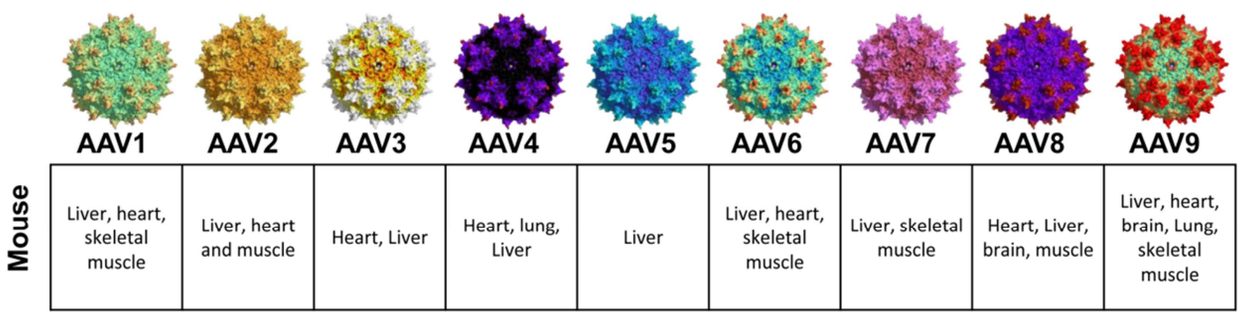

Figure 3. Biodistribution of AAV serotypes 1–9 in mouse.

According to the biodistribution study of AAV following tail vein injection into the mouse, AAV9 has the broadest tissue tropism, demonstrating robust transduction of all tested tissues other than the testes [6] (Figure 3). Moreover, it is the most efficient in reaching the brain, followed by AAV8. On the other hand, AAV7 showed strong tropism for the liver and to a lesser extent for the muscle. Meanwhile, AAV6 had more preference for the heart, in comparison to liver, lung and muscle. AAV4 was found in higher viral copies in the lung, followed by the heart. The rest of the serotypes transduced the selected tissues with lower efficiencies. AAV1 and AAV2 were more prone to reach the liver. In terms of infection kinetics, AAV7 and AAV9 were the fastest in targeting the tissue and showing expression of the reporter gene, luciferase. Meanwhile, AAV3 and AAV4 were the slowest ones. Additionally, among all the serotypes, AAV2, 3, 4 and 5 showed the lowest transduction efficiency.

The first AAV primary receptor that was identified was heparin sulfate proteoglycan (HSPG). It is the receptor that AAV2 and AAV3 bind when infecting cells (Figure 4). Even though AAV6

was shown to have moderate binding affinity for heparin, it does not have the two residues R585 and R588, that participate in AAV2 binding to HSPG. On the other hand, while sequence alignment comparison between AAV1 and AAV6 capsids revealed only a six–amino acid difference, AAV1 is not able to bind heparin. Mutagenesis analysis revealed that amino acid 531 was responsible for providing the heparin binding ability to AAV6 and not to AAV1 [12]. Furthermore, AAV1 binds both α2–3 and α2–6 *N*-linked sialic acid (SIA), same as AAV6. Interestingly, AAV5 also binds α2–3 SIA, although it only shares ~40% homology with capsid serotypes AAV1 and AAV6. Crystallography studies of AAV5 showed differences in the surface loop regions, specifically smaller HI loop and larger VR-VII loop, which are located on the depression wall at the icosahedral 2-fold axis and determine receptor binding, tissue transduction efficiency and antigenic reactivity [13].

AAV4 capsid serotype follows AAV5 in terms of low sequence similarity with the rest of the serotypes and between themselves (53% sequence homology). A study, in which sialic acids were removed from cell surfaces, by neuraminidase treatment, showed that both viruses require SIA for infectivity [14]. However, when cellular glycosylation was inhibited, only treatment with *O*-linked inhibitor decreased binding of AAV4 to cultured cells. Meanwhile, treatment with *N*-linked inhibitors of glycosylation blocked AAV5 binding to the cell surface. Resialylation experiments with neuraminidase-treated red blood cells further confirmed that AAV4 binding to SIA is through α2–3 *O*-linkage, rather than through α2–6 *N* linkage, which is the interaction that AAV5 establishes for the initial infection of a cell.

Still, currently, receptors for AAV7 and AAV8 are unknown. Glycan binding analysis on microarrays revealed that AAV7 and AAV8 did not bind to any of the glycans that commonly bind serotypes AAV1–6 [15]. However, similarly to AAV2 and AAV3, AAV8 and AAV9 interact with the 37/67 kDa laminin receptor (LamR), as a secondary receptor, for efficient internalization and transduction [16]. LamR participates in interactions of extracellular laminin1 with proteases and with the cell; therefore, it is widely distributed among human tissues. Even though, AAV2, 3, 8 and 9 serotypes mediate direct tissue transduction via interaction with the LamR molecule, they significantly differ in their tissue tropism. AAV8 and 9 are able to infect a broader spectrum of tissues, even the brain, compared to serotypes AAV2 and AAV3. This result suggests that the primary receptor or the combination of both receptors is required for viral biodistribution. At the UNC gene therapy center, the Asokan laboratory was able to identify that *N*-linked glycans with terminal galactosyl residues are involved in AAV9 tissue binding and transduction [17]. The high abundance of these glycans in various animal tissues could explain the broad tropism observed after AAV9 systemic administration.

Lastly, the brain is one of the most difficult tissues that AAV can access and infect following systemic administration. The presence of a mature blood–brain barrier constitutes a physical barrier to potential harmful molecules and infectious pathogens. Therefore, most of the AAV serotypes are not able to access the brain without direct intraparenchymal administration. However, AAV9 and AAV8 (to a lesser extent), have the capability to reach the brain following intravenous administration to neonatal or adult mice [18].

Furthermore, the rhesus serotypes AAV10 and AAV11 were found to be sequence homologous and structurally closest to the previously described serotypes AAV8 and AAV4, respectively [19].

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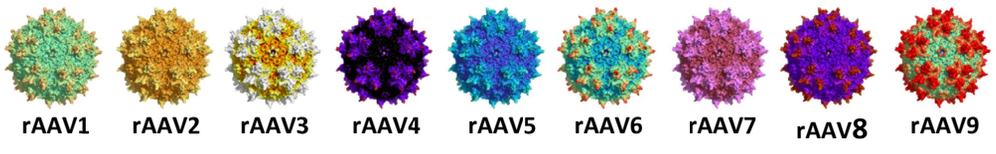
AAV12, which was isolated from a simian adenovirus stock, showed 74% homology with AAV4 and 84% with AAVrh11. However, it does not bind SIA and appears to have strong affinity for human cancer cell lines [20].

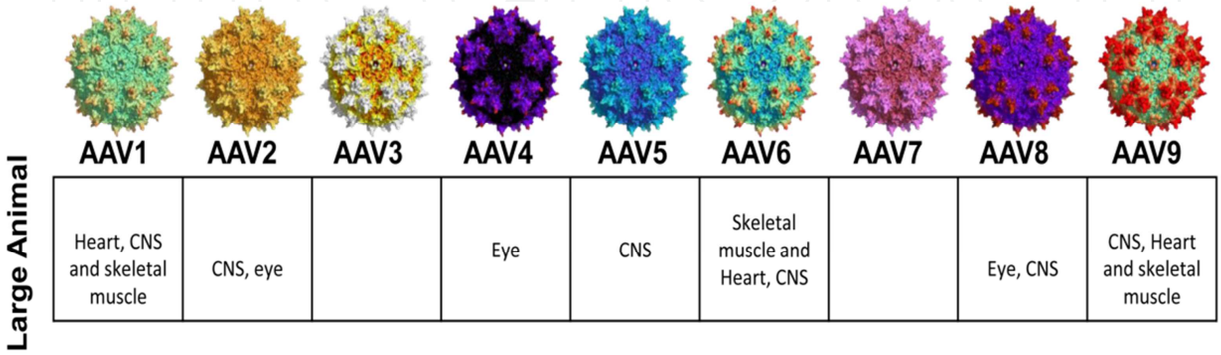


	rAAV1	rAAV2	rAAV3	rAAV4	rAAV5	rAAV6	rAAV7	rAAV8	rAAV9
Primary receptor	N-linked sialic acid	HSPG	HSPG	O-linked sialic acid	N-linked sialic acid	N-linked sialic acid; HSPG	unknown	unknown	N-linked galactose
Secondary receptor	unknown	FGFR1, HGFR, integrins, CD9, LamR	FGFR1, HGFR, LamR	unknown	PDGFR	EGFR	unknown	LamR	LamR

Figure 4. Primary and secondary receptors used for AAV serotypes from 1 to 9 to infect and transduce cell types. HSPG, heparin sulfate proteoglycan; FGFR1 fibroblast growth factor receptor 1; PDGFRB, platelet-derived growth factor receptor beta; HGFR, met/hepatocyte growth factor receptor; EGFR, epidermal growth factor receptor; LamR, laminin receptor.

4. Preclinical studies of rAAV in large animal models

In an effort to translate the rAAV gene therapy to the clinic, preclinical studies for safety, efficient rAAV dosing and capsid transduction, transgene expression and immune responses towards the new transgene and/or the rAAV capsid were performed. In Figure 5, we summarize which serotype has been evaluated for targeting a certain disease in a large animal model, such as nonhuman primate, pig, cat, dog, rabbit and sheep. However, this section will be focused on preclinical studies using large animals for the treatment of hemophilia.



	AAV1	AAV2	AAV3	AAV4	AAV5	AAV6	AAV7	AAV8	AAV9
Large Animal	Heart, CNS and skeletal muscle	CNS, eye		Eye	CNS	Skeletal muscle and Heart, CNS		Eye, CNS	CNS, Heart and skeletal muscle

Figure 5. Diseases treated in large animal models with different AAV serotypes

5. rAAV targeting the liver for hemophilia treatment

Even though rAAV therapies for treating hemophilia in mice produced successful results, their translation into large animals, such as a dog or nonhuman primate, was not straightforward [21]. The vector efficacy does not completely follow a dose–response correlation in large animals, and it is drastically affected in the presence of an immunological response towards the viral capsids. Furthermore, in both mice and dogs, there is no direct correlation between the transgene copy numbers and the expression of the foreign protein. However, treatment of a large animal with the therapy was promising as FIX is a secreted protein and only 1%–2% normal factor IX levels is enough to correct the disease [21].

Therapies for hemophilia B were studied using different routes of rAAV gene therapy administration. Initially, intramuscular delivery of rAAV-CMV-cFIX to hemophilic dogs was pursued. A single administration of the virus generated a therapeutic FIX level in a dose-dependent manner [22]. However, the amount of antibody formation, and therefore, the success of the therapy, directly correlated with the increase in rAAV dose [23]. Considering that FIX is produced within the liver, delivery of the virus through the portal vein was also attempted. Several steps were performed in order to optimize the vector, such as the addition of a liver specific promoter, and testing different doses to determine the optimum for allowing normal levels of FIX in the dog through this route [24–26]. Mount et al. observed sustained levels of FIX between 4% and 12% at doses between 1.2×10^{12} and 3.4×10^{12} vg/kg for over 17 months in three out of four dogs [26]. The fourth dog experienced a transient correction of FIX levels for four weeks but eventually developed neutralizing antibodies against the transgene. In another study, delivery of a hyperfunctional FIX through rAAV-mediated transfer to the liver in hemophilic dogs resulted in 25% and 300% FIX levels for 1×10^{12} and 3×10^{12} vg/kg doses, respectively [27].

Studies performed with nonhuman primates generated variable outcomes, from no detection to detection up to 10% of FIX in the serum. Failure of the gene therapy was attributed to the preexistence of neutralizing antibodies against the transgene [28, 29]. However, direct administration of the rAAV vector to the liver induced some tolerance to the transgene [27, 28]. Another strategy to overcome the success of the therapy due to the presence of neutralizing antibodies consists of administration of the therapy to an early age of the animal, presumably because the immune system is not completely developed [30].

A comparison study of different administration routes for rAAV-FIX delivery on mice revealed that the same dose allowed 3-fold more transgene expression following intrahepatic rather than intramuscular or intravenous delivery [31]. Therefore, it seems that transgene delivery via rAAV virus is more successful when using the liver-directed gene route. When intrahepatic therapy was administered to dogs who suffered from hemophilia A, rAAV8 carrying the canine factor VII cDNA showed long-term correction of the phenotype, with no spontaneous bleeding episodes, no toxicity and no development of inhibitory antibodies towards the viral vector or the transgene [32]. Similarly, liver-directed rAAV-FIX therapy to dogs suffering from hemophilia B, significantly increased FIX activity to 4%–10% and remained stable for more than eight years [33]. However, direct injection of the virus to the muscle resulted in undetectable FIX levels in the dog due to the onset of an immune response.

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6. Clinical trials using rAAV technology

Among the clinical trials reported in clinicaltrials.gov website, which cites ongoing studies all over the world, the United States is still the leading country conducting clinical trials with rAAV gene therapy. In 2010, 47 studies out of 70 were performed in the United States and in 2015, 44 total studies out of 66 have been sponsored by the same country. Since the first registered trial in 2004, a total of 14 studies have been completed, and three terminated prematurely. Furthermore, there are clinical trials in all phases as well as for traditional, not traditional and even recombinant serotypes (Figure 6). Worldwide, there are a total of more than 130 clinical trials testing rAAV gene therapy for the treatment of diseases (http://www.abedia.com/wiley/vectors.php).

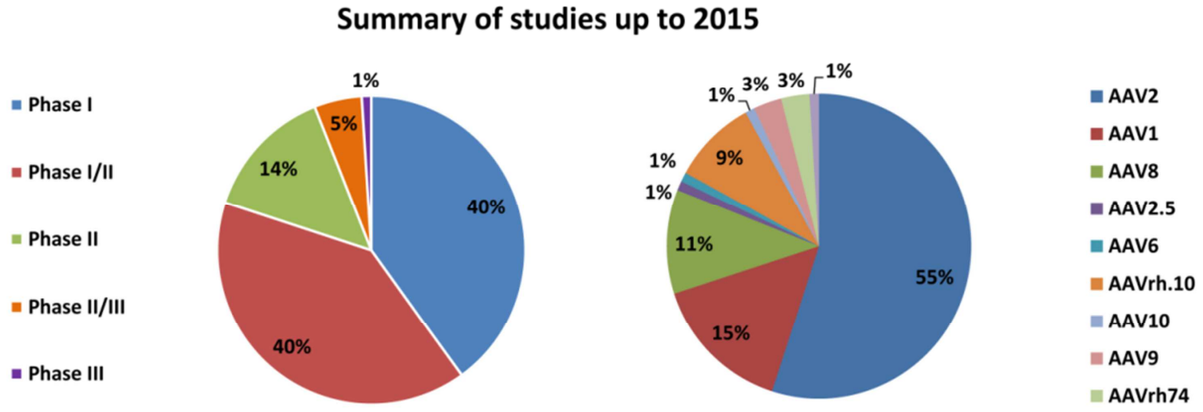

Figure 6. Statistics showing the clinical trials performed with rAAV gene therapy until 2015 according to clinicaltrials.gov. **A,** Classified according to the clinical phase. **B**, Classified according to the rAAV serotype used in the study.

7. rAAV serotypes used in clinical trials

Traditionally, the most common serotype used in clinical trials is AAV2. In 2010, sixty-two clinical trials were performed with rAAV2 vector; meanwhile, the number was reduced to thirty-six, almost half, in 2015 (89% vs. 54%) (Figure 7A). In addition, in 2010, three studies were performed with rAAV1, which increased to 10 in 2015 (4% vs 16%). Interestingly, more uncommon serotypes are acquiring an interest among the scientific community and the spectrum of serotypes being tested is increasing. Five years ago, five out of seventy clinical trials used serotypes other than rAAV1 and rAAV2. On the contrary, now in 2015, eighteen out of the current forty-nine trials are reported in clinicaltrials.org website (7% vs. 37%). For instance, the number of studies using serotype rAAV8 increased from two to seven in a five-year frame (Figure 7B).

The same results were found with the rhesus serotype rAAVrh10; initially, there was one study testing the virus; however, in 2015, six studies have taken place. To note, another rhesus rAAV serotype is being examined: rh74 for duchene muscular dystrophin. Serotypes rAAV5 and

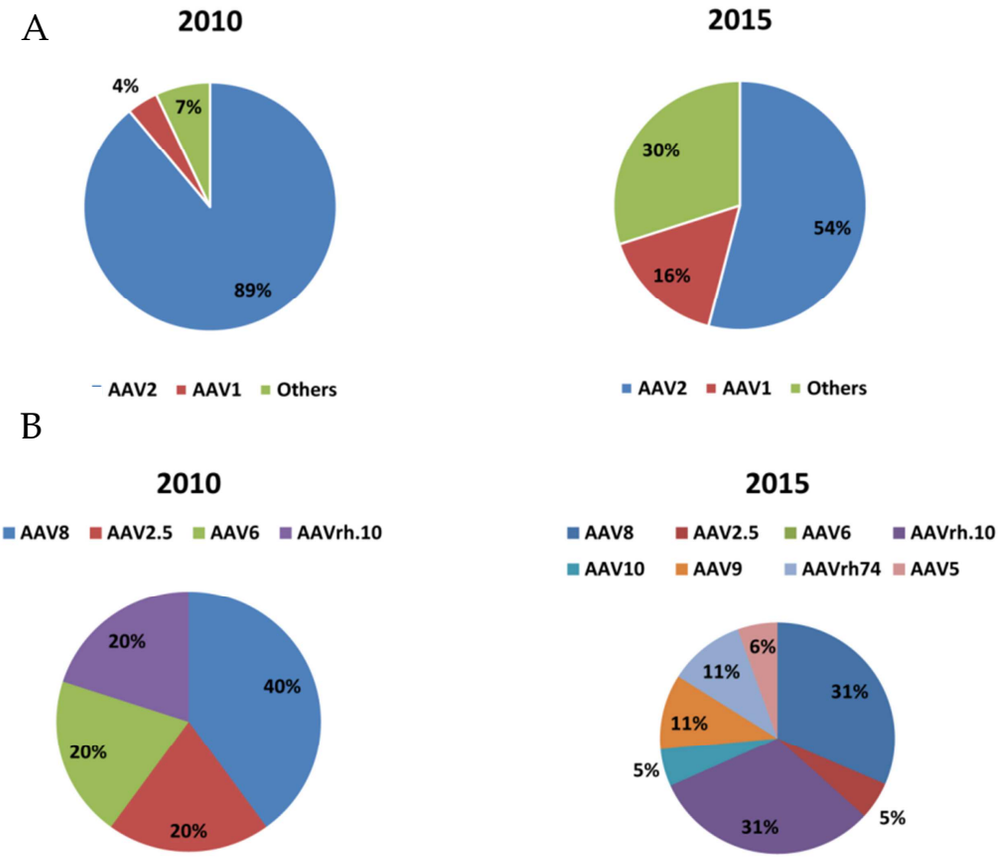

Figure 7. Statistics showing the clinical trials performed with rAAV gene therapy in 2010 and in 2015. **A,** Classified according to the use of traditional serotypes rAAV2 and rAAV1. **B**, Classified according the use of no traditional rAAV serotype.

rAAV9 also were introduced into the list of rAAV viruses in clinical trials. Similarly, phase I/II rAAV10 trial for Sanfilippo type A syndrome started in 2011 and finished in 2014.

As Figure 8A shows, in 2010 a high percentage of the rAAV therapies were in phase I (62%) and a small percentage of the studies (17%) were testing phase I and II on the same trial. In 2015, the number of studies in phase I exclusively was reduced by 20%, compared to studies performed in 2010, and that extra 20% is testing safety and efficacy at the same time (phase I and II, 37%), probably due to the expensive costs of conducting a clinical trial.

Furthermore, the number of studies that were in phase III was reduced, as the therapies started to reach the market. For instance, in October 2012, Glybera became the first rAAV gene therapy to obtain marketing authorization from the European Commission.

Since their discovery in the 1960s as small DNA viruses contaminating cultures of simian and human adenoviruses [2, 34], AAV vectors have been tested in more than a hundred clinical trials. Completed and ongoing trials have consistently confirmed that rAAV vector delivery is safe, well tolerated by humans and efficient in transferring the therapeutic gene. Figure 8B summarizes the spectrum of diseases that have been tested with rAAV gene therapy in 2010

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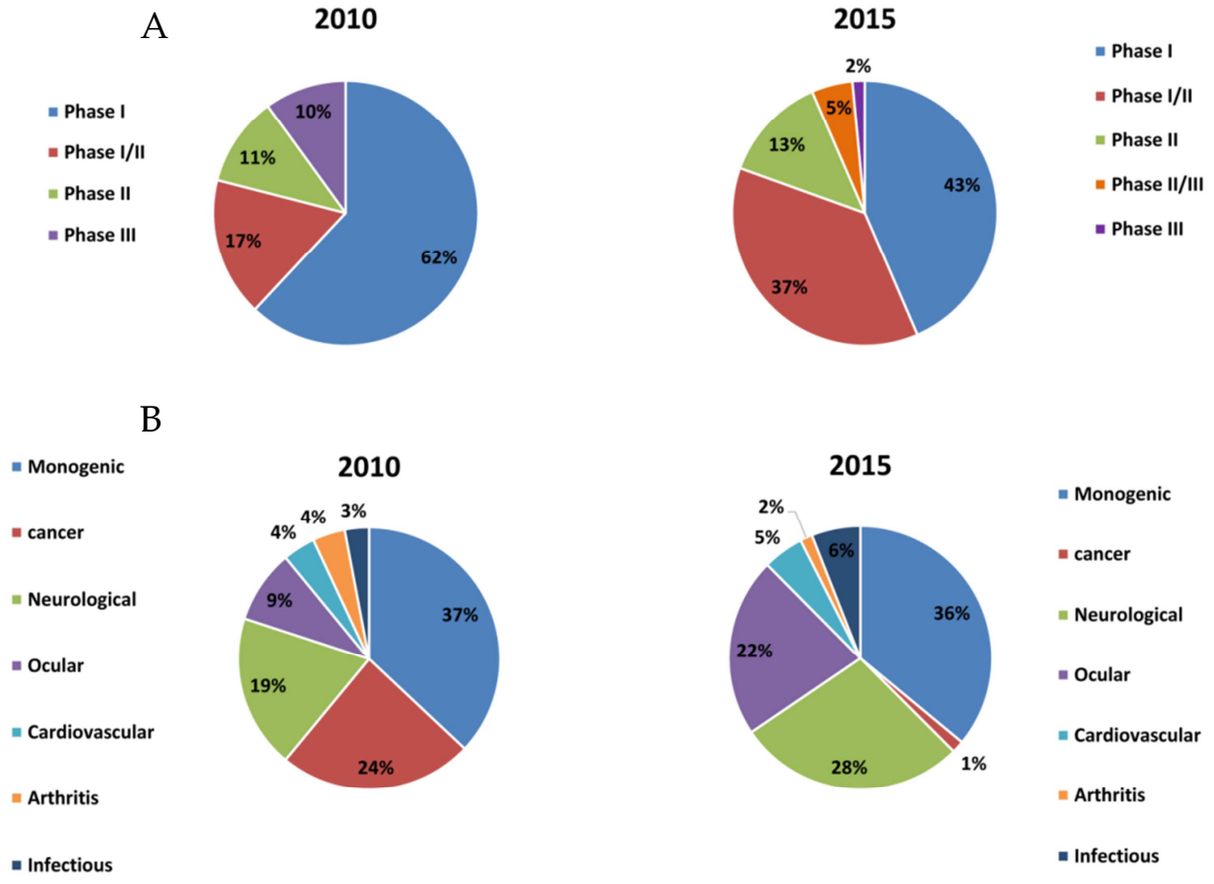

Figure 8. Statistics showing the clinical trials performed with rAAV gene therapy in 2010 and in 2015. **A,** Classified according to clinical phase. **B**, Classified according the treated disease.

and 2015. The statistics show that neurological and ocular diseases are gaining more interest, probably because they both constitute immunological privileged tissues. Figure 9 summarizes the diseases that are being treated with AAV technology, according to the serotype.

As an ocular AAV therapy, two clinical trials have tested rAAV2 therapy for the correction of Leber congenital amaurosis, an autosomal recessive disease that results in blindness. Specifically, patients who participated in these studies received the normal copy of the retinal pigment epithelium-specific 65 (RPE65) gene to correct for the deficient gene. One trial was performed in London and consisted of delivering the gene, the expression of which was driven by an endogenous RPE65 promoter, to adolescent patients [35]. On the other hand, the study performed in Philadelphia delivered the gene in the context of a constitutive promoter, to pediatric and adult patients [36, 37]. Pediatric patients treated in the US resulted in the best improvement in vision, followed by American adults. However, one out of three British patients manifested a visual function improvement. Another trial, sponsored by the University of Pennsylvania, conducted an open-label, dose-escalation phase I study on 15 patients aged between 11 and 30 years. The study examined safety and efficacy. Results showed no toxicity due to the therapy, although some adverse events were observed from the surgery procedure.

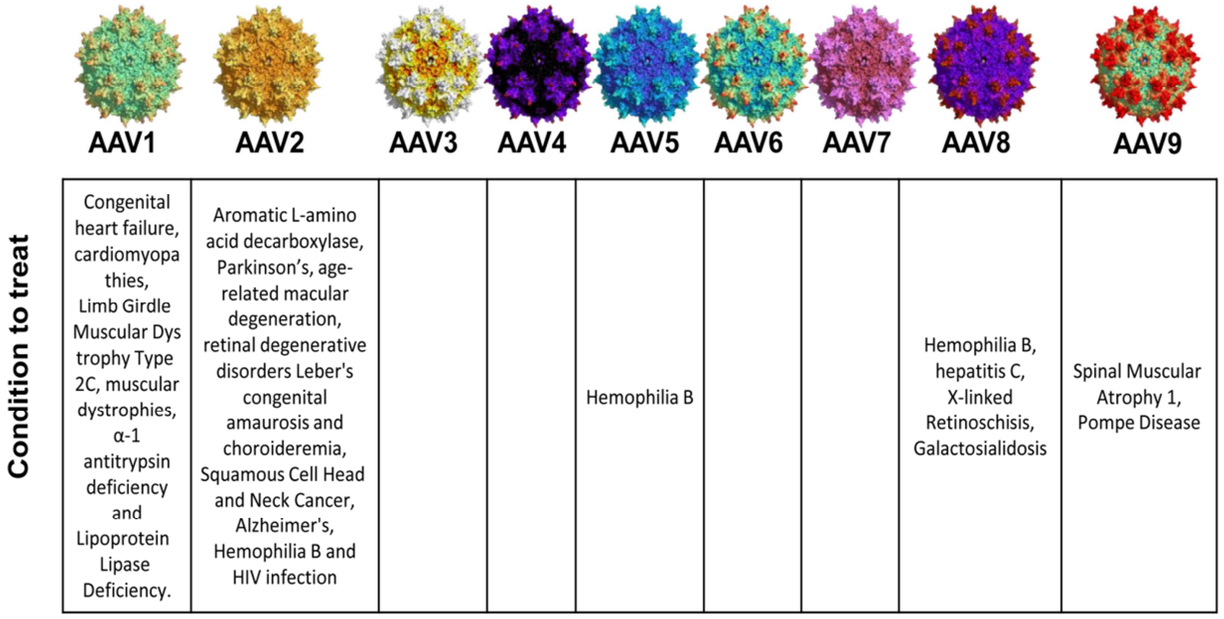

Figure 9. Diseases currently being tested in clinical trials with different rAAV serotypes.

Furthermore, visual function was improved in the 15 patients with a variable degree [38]. However, between 9 and 12 months of gene therapy administration, four of the fifteen patients experienced new pseudo-foveas in the retinal regions, for up to six years [39].

The company Sparks, which is sponsoring the studies in the US, is testing the technology developed at the Children's Hospital of Philadelphia in a phase 3 trial and expects to announce their results in 2015. If the results are promising, it could be the next rAAV gene therapy product to be launched in the market.

Among the brain diseases, Parkinson's treatment was attempted with rAAV gene therapy delivering different transgenes. Administration of glutamic acid decarboxylase (GAD) via rAAV2 produced modest efficacy improvements. Patients were injected with rAAV2 coding for GAD65 and GAD67 in the center of the subthalamic nucleus [40]. Six months following the injection, the unified Parkinson's disease rating scale decreased by 8.1 points, compared with a reduction of 4.7 points that the sham operation group evidenced. Six months later, clinical improvements were still being noticed. However, the results were modest and the protocol had some deviations. For instance, patients who showed no benefit on the primary endpoint were eliminated from the statistical analysis, arguing that the injections were off-target [41]. Administration of aromatic L-amino acid decarboxylase (AADC) gene was tested on a phase I trial that consisted of the treatment of 15 patients with moderate disease [42, 43]. The trial, sponsored by Genzyme, observed only a modest efficacy, results that were confirmed by a second study performed in Japan [44]. Similarly, phase I and II trials with the rAAV2-neurturin (CERE-120) vector from Ceregene failed to show statistically significant improvement in the rAAV-treated group compared with the group that received the sham surgery [45, 46]. As a conclusion of all these different trials, the technology is safe and is promising. However,

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efficacy is modest and does not justify the procedure. Further improvements could be performed, such as modification of the delivery vector, as rAAV1 and rAAV5 are more efficient in transducing the substantia nigra and caudate nucleus than rAAV2. Furthermore, viral dose increase should be considered [41]. On the other hand, long-term improvements were observed during the treatment of Canavan disease [47]. Patients were administered rAAV2 carrying the aspartoaculase gene directly to the brain parenchyma. Five years posttreatment, patients presented slower progression of brain atrophy, fewer frequent seizures and general clinical stabilization. Importantly, no serious adverse events were observed, even when one of the patients was a 3-month-old infant [48].

In a trial testing gene therapy for cardiac disease, patients received different doses (low, medium or high) of the sarcoplasmic reticulum Ca2+-ATPase (SERCA2a) gene via rAAV1 [49]. Six months following the percutaneous intracoronary infusion of the virus, several clinical parameters, such as walk test, peak maximum oxygen consumption, left ventricular end-systolic volume, cardiovascular events and time to clinical events were stabilized or even improved. Currently, a phase 2b trial is ongoing, which would test a larger patient population (NCT01643330).

The first clinical trial for rAAV gene therapy that reached the market was the product Glybera® (alipogene tiparvovec), an rAAV1 vector delivering a lipoprotein lipase variant (LPLS447X) for the treatment of lipoprotein lipase deficiency (LPLD). Lipoprotein lipase is a secreted enzyme produced by the skeletal muscle and adipose tissue. Its function involves the metabolism of triglycerides, chylomicrons and very low-density lipoproteins. Three clinical trials showed that Glybera is safe and efficient for the treatment of LPLD. In the first trial, two doses of rAAV1_LPLS447X were studied: low and high [50]. Nevertheless, none of the doses resulted in a permanent decrease in triglyceride levels. There was only a transient reduction, possibly due to the development of an immune response. The second clinical trial received the therapy in combination with an immunosuppressive regimen [51]. Similar to the first clinical trial, the effects of the therapy were only transient in the beginning. However, improvements were observed after two years posttreatment, such as tolerance to certain foods, changes in the blood lipid content and a decreased frequency of pancreatitis. Due to a discrepancy in the clinical outcomes and plasma triglycerides levels, a third trial was set with predetermined parameters to measure, as incidence frequency of abdominal pain, pancreatitis and chylomicron plasma clearance [52]. Five newly treated patients evidenced a reduction of the parameters and an improved quality of life for two years following administration.

These results, combined with the ones obtained from the reanalysis of 22 of the 27 previously treated patients, confirmed the therapeutic benefits of therapy and granted its approval to the market by the European Commission in November 2012.

8. Clinical trials for hemophilia B treatment

Hemophilia B is an X-linked recessive disorder, which originated from mutations within the gene that encodes the coagulation factor IX. Therefore, patients whose functional FIX levels

are 1% of normal levels will bleed into the joint and muscle tissues. Bleeding in the brain could result in fatal death. If FIX levels are between 1% and 5%, the individual will experience a reduced number of bleeding incidents and a moderate phenotype of the disease. Any FIX levels above 5% will allow the person to have a normal life [53]. The only available treatment is protein replacement therapy, which requires regular intravenous injections and is expensive. Therefore, novel and permanent therapies/treatments are urgent. rAAV gene therapy currently constitutes a promising approach for the treatment of several diseases, including hemophilia B.

Based on animal studies that were described in the previous section, four clinical trials have been initiated. The first study administered rAAV carrying FIX gene into three patients by intramuscular injection. Despite the presence of preexistent high titer of neutralizing antibodies against capsid rAAV, strong transgene expression was observed in the muscle, even after 10 months of injection. However, levels of factor IX in circulation were less than 1–2% in most cases, even at the highest tested dose. Toxicity was not observed [54, 55].

The second trial conducted by the University of Pennsylvania infused the virus through the hepatic artery into seven patients. The rational in this protocol considered that FIX is a secreted protein and once it is produced and reaches the bloodstream; it can be distributed throughout the body.

Even though levels of FIX resulted higher than 5% after injecting an intermediate or high viral dose, the therapeutic effect was only transient (up to eight weeks), due to the development of a strong cytotoxic T response, which destroyed the transfected hepatocytes and thus hampered the production of FIX [56].

The third trial was designed in order to increase FIX expression production as well as to circumvent the possibility of a humoral response that could interfere with the success of the therapy [57]. To reach the first goal, they developed a codon optimized FIX gene that also delivered the gene in the context of self-complementary rAAV, which provides substantially higher levels of transgene expression rather than delivering the WT gene with single-stranded rAAV. In order to reduce antibody neutralization, the viral genome cassette was packed in rAAV8 capsid, as it has lower seroprevalence in humans and a high tropism for the liver. The virus was administered directly in the peripheral vein in six patients, and all of them developed 1–6% levels of factor IX expression in the first four months as well as for at least three years. There was no modification on the levels of neutralizing antibodies. However, transient elevations in serum liver enzymes, possibly as a result of a cellular immune response to the rAAV8 capsid, were observed in the three patients who received the high viral dose.

Recently, Baxter's laboratories launched a clinical trial to test the safety and efficacy of a self-complimentary rAAV8 vector carrying a mutant FIX sequence (BAX 335), created and preclinically tested at the UNC gene therapy center [58]. The FIX sequence had a single amino acid change at position 338, which substantially increased the levels of circulating FIX protein. A more effective rAAV delivery vehicle allowed the administration of lower viral doses with the same efficacy as previous tested virus, but without the associated toxicity. In this trial, sixteen adult patients were treated in different centers around the US. Updates on the trial

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were presented at the 8th Annual Congress of the European Association for Haemophilia and Allied Disorders this year in Finland. At this time, patients who received the highest dose did not develop inhibitory antibodies, reached FIX protein activity of 10% or more and did not manifest bleeding events. However, one of the patients experienced elevated levels of liver enzymes probably due to an immune response.

Nowadays, investigators are still developing better strategies to overcome the immunological response; currently, there are six trials evaluating safety and efficacy, including BAX 335.

9. rAAV and gene editing technology

Several genome editing tools have emerged recently in an attempt to correct the genetic cause(s) of a disease. These technologies rely on two components: a sequence-specific DNA-binding domain and a nuclease [59]. The procedure consists of several steps: (1) recognition of a targeted DNA sequence, (2) double stranded cut and (3) stimulation of a cellular repair mechanism to correct the DNA damage, which includes homologous recombination [60].

This technology allows for modifying a coding sequence, the epigenome, transcriptional activator/repressor as well as a regulatory element such as transcription factors, recombinases, transposases, and more. When targeting a particular gene, these technologies generate deletions, insertions or mutations of the gene, which may be useful to elucidate the gene function, or to generate cell lines with the null phenotype, or even to model a specific genetic condition for its study. Three different systems are currently available: zinc-finger nucleases (ZFN), TALEN nucleases and CRISPR/Cas9 [60].

Zinc-finger nucleases are a common type of DNA-binding motif found in eukaryotes and therefore, in the human genome. Usually, the DNA binding domain in the zinc-finger nuclease recognizes three base pairs in the DNA sequence. However, researchers have engineered the domain in order to detect and bind any defined DNA sequence of 9 to 18 bps in length, allowing the targeting of up to 68 billion bp of DNA [61].

Even though these technologies are very promising, an optimal delivery vehicle of the gene editing system needs to be developed. rAAV has the potential to deliver nucleases in vitro and in vivo and also has the potential to induce homologous recombination in the cell that infects, further enhancing the homologous recombination efficiency by 1000-fold [62–64].

In 2011, High et al. showed the generation of a ZFN system capable of cleaving F9 intron 1 and inducing homology-directed repair in the human hepatocyte Hep3B cell line. As proof of principle, the system induced up to 17% stable integration of a novel restriction enzyme site into the F9 locus. Furthermore, intraperitoneal administration of a ZFN system, which specifically targets F9, via rAAV8 delivery, in conjunction with an rAAV8 vector carrying a corrective F9 complementary DNA cassette into a humanized neonatal mouse model of hemophilia B, resulted in 1%–3% specific targeting of mouse liver. However, this mouse at two days old produced 2–3% normal F9 levels, enough amount to convert severe to mild hemophilia [65]. In 2013, they tested the same technology in a young adult mouse (8–10 weeks

old), in which hepatocyte proliferation is slow as the liver already reached its maturity [66]. In theory, younger mice should show higher levels of gene correction, compared to older mice, as mice age affects the rate of homologous recombination, which is essential for genome editing to occur. In this publication, even though adult mice showed limited hepatocyte proliferation, following AAV injection, mice experienced a 5-fold increase in FIX expression, compared to the previous study. Moreover, when they tested the technology in even older mice, 7–8 months old, FIX levels were extremely low. Investigators argue that the discrepancies in FIX levels between neonate and adult mice could be attributable to the loss of rAAV vector genomes during liver development and/or different promoter activity. Furthermore, when they switched the use of homodimer nucleases to heterodimeric ZFN, nonespecific ZFN cleavage was observed without the loss of FIX expression.

Additionally, ZFN technology is currently being investigated in clinical trials for the treatment of HIV. Basically, the therapy consists of ex vivo permanent modification of patient T cells to knock down the HIV entry receptor CCR5 and autologous administration of the recombinant cells back to the patient. This clinical trial is sponsored by Sangamo Biosciences, the same company that collaborated with D. High for the in vivo targeting of hemophilia B mouse with rAAV-ZFN platform. In 2014, the company released an announcement for the first IND to test ZFN genome-editing platform in hemophilia A patients.

Even though these studies performed by Dr. High's laboratory and Sangamo Biosciences showed potential for in vivo gene editing via rAAV delivery, especially for diseases which do not allow ex vivo manipulation of target cells such as hemophilia B, the technology has several issues to address before being considered efficient and safe for treating human patients. First of all, we should consider all the challenges associated with rAAV delivery in vivo, such as the development of a cytotoxic T cell and/or neutralizing antibody responses and exclusively targeting of the tissue to correct with high efficiency. Furthermore, in order for this therapy to work efficiently, each cell needs to receive the two viruses at the same time, a condition that is possible but with a much lower probability to occur, and even if it takes place, the individual would be exposed to higher doses of rAAV8, which enhance the probability of inducing an immune response. Given that homologous recombination repair mainly takes place during the S phase of the cell cycle, gene editing is limited to be successful only in young patients, unless it is combined with molecules/drugs that boost cell division. Importantly, off-target double-stranded DNA breaks pose the possibility of inducing vector integration and/or undesired mutations and consequently, inducing oncogenesis, cell death and/or genetic diseases. This last possible issue could be solved by using self-inactivating viruses. Finally, the ZFN rAAV-mediated technology is still in the early phase of development, so far it has proven its potential for permanently correcting monogenetic diseases. However, considering that (1) rAAV gene therapy has shown great promise in the treatment of hemophilia B and (2) very low levels of FIX are enough to prevent bleeding and allow the person to have a normal life, in vivo gene editing technology seems too risky and unnecessary to pursue for the treatment of hemophilia diseases.

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10. iPSC and rAAV

iPSC technologies have gained special interest since their discovery in 2006 by Takahashi and Yamanaka [67]. The generation of iPSC has several applications. One of the most important applications consists of the generation of: (1) pluripotent stem cells from a fully differentiated patient cell or (2) a specific human cell that is scarce or not accessible to the scientific population, from a healthy or diseased individual, following differentiation of the pluripotent stem cell. Moreover, sometimes, a personalized treatment is required or a diverse population cell sample is needed for testing the efficacy of a therapeutic technology, such as rAAV. For instance, the common practice is to reprogram patient-derived fibroblasts into a specific cell type that is affected by a disease. Some attempts have been performed to reprogram fibroblasts of patients suffering from retinal diseases into iPSC and finally differentiate the pluripotent stem cells into retinal pigment epithelial (RPE) cells that manifest the diseased phenotype [68, 69]. Following the validation of iPSC and then RPE cells, a panel of rAAV serotypes could be tested for their efficiency to transduce the cells and the most effective ones could be chosen for delivering the healthy gene copy in order to re-establish normal cellular phenotype [70].

Another approach for inducing iPSC development has been tried, but this time, using an rAAV system rather than a retrovirus. IPSC generated by Takahashi and Yamanaka's original protocol made use of retroviruses to deliver Oct3/4, Sox2, Klf4 and c-Myc. Even though the approach resulted in the generation of pluripotent stem cells from mouse embryonic or adult fibroblast cultures, still the efficiency was extremely low, the presence of c-Myc oncogene significantly increased the incidence of tumorigenicity and the use of retrovirus posed the threat of integration into the genome. Several new strategies have been developed, including the use of rAAV [69, 71]. Considering the advantages of using rAAV for gene delivery, such as long-term transgene expression for efficient reprogramming of mature cells as well as safety and efficacy as a gene delivery vehicle in the clinic; researchers have attempted their use in the reprogramming of fully differentiated fibroblasts as well as adipose-derived mesenchymal stem cells. However, both studies observed frequent rAAV integration into the host genome of iPSC cells when the iPSC were generated from nondividing cells. Integration events were independent of the rAAV vector, cell type and amount of virus. Both studies concluded that there is a certain degree of incompatibility between iPSC generation and the use of rAAV vectors, although reprogramming does not require an integration event. Furthermore, like retrovirus-mediated cell reprogramming, rAAV-mediated iPSC generation resulted in reprogramming transgene silencing, which affects the quality of the induced pluripotent stem cells that could be generated. Therefore, if the integration events are tightly controlled, which is feasible, and if the epigenetic mechanisms of rAAV silencing are discovered, rAAV technology could result in a safer mechanism for inducing pluripotent stem cells and consequently, increasing the chances of being applicable to the clinic.

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