Review

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Review

Challenges in Humoral Immune Response to Adeno-Associated Viruses Determination

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Abstract: Adeno-associated viruses (AAVs) are non-pathogenic, replication-deficient viruses that have gained widespread attention for their application as gene therapy vectors. While these vectors offer high transduction efficiency and long-term gene expression, the host immune response poses a significant challenge to their clinical success. This review focuses on the obstacles of evaluating humoral response to AAVs. We discuss the problems with the validation of *in vitro* tests, and the possible approaches to overcome them. Using published data of neutralizing titers of AAV serotypes, we built the first antigenic maps of AAV in order to visualize the antigenic relationships between varying serotypes.

Keywords: AAV; neutralizing antibodies; antigenic cartography; anti-AAV antibodies; humanized models; NHP models; mice models

1. Introduction

Adeno-associated viral vectors are thought to be a perspective delivery platform for gene therapies due to their high transduction and long-term expression efficiency in different cell types, non-pathogenic nature, and low immune responses in humans [1,2]. AAVs are unknown to induce any human diseases [3], however, they naturally circulate in certain host animals. People may be spontaneously infected by AAVs during life, which can lead to increased levels of AAV-specific serum antibodies [4,5]. As a result, up to 80% of the human population has neutralizing antibodies to AAVs [6], with humoral response to AAV2 being most prevalent [7]. T-cell-mediated immune responses to AAV are rarely detected in treatment-naive individuals because of low sensitivity tests or other reasons [8–12]. However, this review is focused on humoral responses to AAV and methods of evaluating antibody titers to AAV.

Pre-existing humoral immune response is thought to be a deleterious factor for AAV-based gene therapy because of the neutralization vector caused by specific serum antibodies [13–15]. A high level of pre-existing anti-AAV antibodies may promote phagocytosis and complement activation [14]. Phagocytized vectors are not processed to transgene expression [16]. As a result, a higher dose of drug is required to achieve therapeutic goals. According to the FDA, high titers of anti-AAV antibodies are an exclusion criteria in the gene therapy clinical trials in which systemic administration is used [17].

Over 30 clinical studies of AAV-based gene therapy (GT) that reported anti-AAV serum antibody titers were analyzed in this study. We found that cutoff titer values varied from study to study, regardless of local or systemic vector administration, the method of measuring titers, or the serotype selected for GT delivery. However, in order to fairly compare cutoff titers from different clinical trials, antibody titer tests need to have been conducted in similar conditions. We found that

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the protocols of these assays are often not detailed enough in descriptions of clinical trials or scientific papers. The differences in providing these assays have direct influence on the titer values.

The correct determination of anti-AAV titer is important not only for definition of the exclusion criteria for GT, but also when comparing humoral responses to different serotypes of AAV. A serotype-switching approach by using low cross-reactive wild-type AAVs or synthetic AAVs may be used to overcome a preexisting humoral response [18]. In such cases, direct comparison of sera antibody activity against serotypes of interest is paramount. ELISA and *in vitro* neutralization assays with permissive cell lines are the most common methods used to determine the level of anti-AAV antibody response [19]. Whereas ELISA is relatively easy to standardize, *in vitro* neutralization assays have limitations for AAV. Here, we discuss the theoretical basement of the methodological problems of modern *in vitro* assays. Particularly, we show why in *in vitro* neutralization assays the ratio of full and empty capsids is important, how the ratio of serum antibodies and used viral particles influences the result, and why the differences in transduction efficiency of cell lines between AAV serotypes are a crucial problem with this assay [20,21]. Some recommendations to overcome these problems are proposed.

The other approach to determining humoral response to AAV is to use animal models with a humanized immune system. Regardless of the obvious difficulties in the usage of animals, there is a possibility for AAVs to transduce different cell types in the whole organism and interact with specific antibodies or immune cells [22]. In this paper, we have reviewed animal models that are used to study human-like humoral responses to AAVs with subsequent *in vitro* neutralization assays. Moreover, we describe an animal model approach that allows the complete avoidance of *in vitro* neutralization assays for the determination of human sera neutralization titers to AAV.

Although animal models have several advanced compared to *in vitro* assays, the last one is more suitable for screening assays and more easy to standardize [23]. The previously listed disadvantages of most current *in vitro* assays influence the level of transduced target cells, which are used to evaluate the neutralization titers. As a result, different neutralization titers reflect not the optimized conditions of the assay, but the antigenic characteristics of different serotypes. However, if antibody titers to different AAV serotypes are obtained in similar conditions, their antigenic characteristics can be evaluated and visualized with various methods, such as antigenic cartography [24]. In the case of AAV serotypes, such maps may be useful in order to reveal the direction in which novel synthetic AAV synthesis should be taken to create AAV that will antigenically differ from the wild type, to which pre-existing immunity is often present. As far as we know, no AAV antigenic maps have been created yet. Here, we constructed an AAV antigenic map using previously published neutralization titer data.

The present review discusses the pros and cons of current *in vitro* assays and animal models to detect human humoral immune responses to AAV and proposes possible improvements. Overcoming limitations of current test systems will allow for better analysis and comparison of data in order to overcome pre-existing humoral responses. This makes the review relevant to researchers and clinicians seeking to enhance the safety and efficacy of gene therapies.

2. Anti-AAV Abs Generation

AAVs transduce a wide spectrum of target cells, including antigen-presenting cells (APCs) [25,26]. In *in vitro* experiments, AAVs were uptaken predominantly by monocyte-derived dendritic cells (moDCs) and by monocytes, conventional and plasmacytoid DCs, and neutrophils to a lesser degree [14]. AAV proposed PAMPs, such as capsid proteins, and CpG in viral genomes [27], interact with PRRs (TLR2, endosomal TLR9, respectively) on or within the cells that trigger an inflammatory reaction and maturation of the dendritic cells, their migration to the lymph nodes [8] and presentation to naive CD8+ or CD4+ T cells. Jamie L. Shirley et al. provided some insights into this process. They showed that activation of TLR9 on pDCs, in which it is highly expressed, led to type 1 interferon production that, in combination with costimulation of CD40L, activated maturation of conventional DCs (cDCs) and presentation capsid antigens in their MHC I [28]. After endocytosis by target cells or APCs, AAVs undergo proteasomal degradation following endosomal escape [29], capsid

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phosphorylation, and ubiquitination [28]. As a result, capsid antigens are presented onto MHC class I or cross-presented onto MHC II. Activated APCs trigger specific T cell response. Activated CD4+ Tfh cells induce B cell activation and antibody secretion. Also, in *in vitro* and in mouse model, it was shown that AAV capsid triggers IL-1β– and IL-6–dependent B cell differentiation and specific IgM and IgGs secretion [30].

In fact, the process of humoral response to AAVs induction is not well studied. There are certain challenges with studying this process due to the absence of an acute inflammation stage after infection with AAVs in humans. However, the presence of a humoral response to AAVs was repeatedly shown, and in adults the level of this response is higher than in children [5]. The level of pre-existing anti-AAV Abs is crucial in AAV-based gene therapies. For example, recently, it was proven *in vitro* that high titers of anti-AAV NAbs trigger complement activation that may determine thrombotic microangiopathy [14].

3. Anti-AAV Antibody Titers as Exclusion Criteria in Clinical Trials

Pre-existing AAV-specific antibodies hamper efficient transduction of AAV and potentially lead to decreased delivery of the gene therapy candidate [6]. No correlation has been shown between anti-AAV Ab levels and adverse events in gene therapy (GT) studies [31]. However, that may be explained by the fact that, in at least 45 percent of the U.S. Food and Drug Administration (FDA)-approved GTs with systemic injection of AAV, patients with pre-existing Abs to AAV are often excluded from clinical trials [32]. The enrollment criteria have been set at either <1:10 anti-AAV NAb titer or <1:100 total anti-AAV-IgG titer [16].

Exclusion of patients from clinical trials with high levels of anti-AAV antibodies greatly depends on the therapeutic area. The deleterious impact of pre-existing antibodies on the therapeutic effect is expected to be more significant when the administration is systemic, as compared to local administration [32]. A meta-analysis by Hau Kiu Edna Au et. al. (2022) showed that almost 90% of blood disease studies exclude patients with pre-existing antibodies, while only <10% of eye and 21% of CNS disease studies exclude such patients [18]. However, FDA guidance recommends exclusion of seropositive patients for both local and systemic administration [33–36].

Titer exclusion criteria differ between studies. In Table 1, we summarized this data from various clinical studies. Trials were extracted from the U.S National Library of Medicine database (ClinicalTrials.gov), the largest clinical trials database to date, following the keyword search for "AAV," using a cut-off of 23 July 2021. Only studies with exclusion criteria for the presence of Nabs in AAV were selected. It should be noted that these titers seemingly do not show absolute titers of ADA (Anti-Drug Antibody).

Table 1. Selected examples of over 30 clinical studies employing AAV.

Disease	Status	Phase	Exclusion Criteria and Method of Detection Anti-AAV Abs	Administration	Sponsor	ClinicalTrials.gov ID
			AAV9			
Muscular Atrophy Type 1	COMPLETED	Phase 1	binding antibody titers >1:50, ELISA	Intravenous	Novartis (Novartis Gene Therapies)	NCT02122952
MPS IIIA	ACTIVE, NOT RECRUITING	Phase 2 Phase 3	binding antibody titers ≥ 1:100, ELISA	Intravenous	Ultragenyx Pharmaceutical Inc	NCT02716246
Mucopolysaccharidosis (MPS) IIIB (MPSIIIB)	TERMINATED	Phase 1 Phase 2	binding antibody titers ≥ 1:100, ELISA	Intravenous	Abeona Therapeutics, Inc	NCT03315182
Late Infantile Neuronal Ceroid Lipofuscinosis 6 (vLINCL6)	COMPLETED	Phase 1 Phase 2	binding antibody titers > 1:50, ELISA	Intrathecally into the lumbar spinal cord region	Amicus Therapeutics	NCT02725580

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AAVrh10						
Late Infantile Krabbe Disease Treated Previously With HSCT (REKLAIM)	RECRUITING	Phase 1 Phase 2	binding antibody titers >1:100, ELISA *This criteria will not apply to children screened before they have received HSCT or for children who sign the inform consent within 60 days from HSCT.	Intravenous	Forge Biologics, Inc	NCT05739643
Alpha-1 Antitrypsin (A1AT)	COMPLETED	Phase 1 Phase 2	neutralizing antibody titer ≥ 1:5, neutralizing Ab	Intravenous or intrapleural	Adverum Biotechnologies, Inc.	NCT02168686
Hemophilia B	TERMINATED	Phase 1 Phase 2	neutralizing antibody titer > 1:5, neutralizing Ab	Intravenous	Ultragenyx Pharmaceutical Inc	NCT02618915
AAV2						
Advanced Parkinson s Disease	COMPLETED	Phase 1	total antibody titer >1000, ELISA	Intracerebral (Bilateral Stereotactic Convection-Enhanced Delivery)	National Institute of Neurological Disorders and Stroke (NINDS)	NCT01621581
Aromatic L-amino Acid Decarboxylase (AADC) Deficiency	COMPLETED	Phase 2	neutralizing antibody titer over 1,200 folds or an ELISA OD over 1 cannot be recruited into this trial.	Intracerebral	National Taiwan University Hospital	NCT02926066
Leber congenital amaurosis (LCA)	ACTIVE, NOT RECRUITING	Phase 1	AAV antibody titers greater than two standard deviations above normal at baseline;	Subretinal	University of Pennsylvania	NCT00481546
Hemophilia B	TERMINATED	Phase 1	Presence of neutralizing antibodies AAV2/6 vector	Intravenous	Sangamo Therapeutics	NCT02695160
AAV8						
Hemophilia B	TERMINATED	Phase 1	neutralizing antibody titer > 1:5	Intravenous	Spark Therapeutics, Inc.	NCT01620801
Late Onset Pompe Disease (FORTIS)	RECRUITING	Phase 1 Phase 2	neutralizing antibody titer > 1:20	Intravenous	Astellas Gene Therapies	NCT04174105
Homozygous Familial Hypercholesterolemia (HoFH)	TERMINATED	Phase 1 Phase 2	neutralizing antibody titer > 1:10	Intravenous	REGENXBIO Inc	NCT02651675
Hemophilia B	TERMINATED	Phase 1 Phase 2	neutralizing antibody titers ≥ 1:5	Intravenous	Baxalta now part of Shire	NCT04394286
Hemophilia B	ACTIVE, NOT RECRUITING	Phase 1	Detectable antibodies reactive with AAV8	Intravenous	St. Jude Children's Research Hospital	NCT00979238
cocaine use disorder	RECRUITING	Phase 1	Patientss how detectable pre-existing immunity to the AAV8 capsid as measured by AAV8 transduction inhibition and AAV8 total antibodies.	Intravenous	W. Michael Hooten	NCT04884594
The Human Immunodeficiency Virus (HIV)	ACTIVE, NOT RECRUITING	Phase 1	titer of pre-existing antibodies to capsid > 1:90	Intramuscular	National Institute of Allergy and	NCT03374202

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					Infectious Diseases (NIAID)	
Hemophilia B	TERMINATED	Phase 1	neutralizing antibody titer > 1:5	Intravenous	Spark Therapeutics, Inc.	NCT01620801
			AAVrh74			
Limb-Girdle Muscular Dystrophy, Type 2D (LGMD2D)	COMPLETED	Phase 1 Phase 2	binding antibody titers ≥ 1:50, ELISA	Isolated limb infusion (ILI)	Sarepta Therapeutics, Inc.	NCT01976091
Dysferlinopathies	COMPLETED	Phase 1	binding antibody titers > 1:50, ELISA	Extensor digitorum brevis muscle (EDB)	Sarepta Therapeutics, Inc.	NCT02710500
Duchenne Muscular Dystrophy	COMPLETED	Phase 1	binding antibody titers ≥ 1:50, ELISA	Extensor Digitorum Brevis (EDB) muscle	Jerry R. Mendell	NCT02376816
			AAV5			
Hemophilia A	ACTIVE, NOT RECRUITING	Phase 1 Phase 2	Absence of pre-existing antibodies against the AAV5 vector capsid, measured by total AAV5 antibody ELISA	Intravenous	BioMarin Pharmaceutical	NCT03520712
Hemophilia B	COMPLETED	Phase 1 Phase 2	Neutralizing antibodies against AAV5 at Visit 1	Intravenous	CSL Behring	NCT02396342
Arthritis	UNKNOWN STATUS	Phase 1	Presence of neutralising antibody (Nab) titers against adeno-associated virus type 5 (AAV5) and/or hIFN-β.	Intra-articular	Arthrogen	NCT02727764
Haemophilia A	ACTIVE, NOT RECRUITING	Phase 1 Phase 2	Detectable pre-existing immunity to the AAV5 capsid as measured by AAV5 transduction inhibition or AAV5 total antibodies	Intravenous	BioMarin Pharmaceutical	NCT02576795
			AAV1			
CMT1A	SUSPENDED Vector has not been produced	Phase 1 Phase 2	binding antibody titers ≥ 1:50, ELISA	Intramuscular	Nationwide Children's Hospital	NCT03520751
Duchenne Muscular Dystrophy	COMPLETED	Phase 1 Phase 2	binding antibody titers > 1:50, ELISA	Intramuscular	Jerry R. Mendell	NCT02354781
Heart Failure	TERMINATED	Phase 2	neutralizing antibody titers ≥ 1:2	Intracoronary	Assistance Publique - Hôpitaux de Paris	NCT01966887
Advanced Heart Failure	COMPLETED	Phase 2	neutralizing antibody titers ≥ 1:2	Intracoronary	Celladon Corporation	NCT01643330
Limb Girdle Muscular Dystrophy Type 2C	COMPLETED	Phase 1	Pre-injection neutralizing anti-AAV1 antibodies titer superior or equal to 1/800.	Intramuscular	Genethon	NCT01344798
Becker Muscular Dystrophy and Sporadic Inclusion Body	COMPLETED	Phase 1	neutralizing antibody titers ≥ 1:1600, ELISA	Intramuscular	Nationwide Children's Hospital	NCT01519349

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Heart Failure	RECRUITING	Phase 1	Anti-AAV1 neutralizing antibodies	Antegrade epicardial coronary artery infusion	Sardocor Corp.	NCT06061549
Others						
Hemophilia A	ACTIVE, NOT RECRUITING	Phase 3	Anti-AAV6 neutralizing antibodies	Intravenous	Pfizer	NCT04370054
Hemophilia B	COMPLETED	Phase 2	Neutralizing antibodies reactive with AAV-Spark100 above and/or below a defined titre	Intravenous	Pfizer	NCT02484092
Hemophilia A	ACTIVE, NOT RECRUITING	Phase 1 Phase 2	Detectable antibodies reactive with AAVhu37capsid.	Intravenous	Bayer	NCT03588299
Hemophilia A	COMPLETED	Phase 1 Phase 2	Detectable antibodies reactive with AAV-Spark200 capsid	Intravenous	Spark Therapeutics, Inc.	NCT03003533
Hemophilia A	COMPLETED	Phase 1 Phase 2	Detectable antibodies reactive with AAV-Spark capsid	Intravenous	Spark Therapeutics, Inc.	NCT03734588
Fabry Disease	ACTIVE, NOT RECRUITING	Phase 1 Phase 2	Presence of high titer neutralizing antibody to 4D-310 capsid, or presence of high antibody titer to AGA	Intravenous	4D Molecular Therapeutics	NCT04519749

Table 1 shows heterogeneity in the approaches to anti-AAV determination: some studies use ELISA to measure binding antibodies, while others use neutralization tests with cell cultures to measure NAbs or transduction inhibition tests. Titer values vary from 1:2 to 1:20 for neutralizing antibody titers and from 1:50 to 1:1600 for binding antibody titers, which confirms the lack of uniform standards for this parameter. The insufficiently precise wording in exclusion criteria in a number of clinical studies is also questionable.

In most clinical trials presented in Table 1, there is no information on how titers were defined. Apparently, it is a serum dilution that exceeds the cut-point value, which was also not defined. By the recommendation of the FDA, the cut-point of the level of ADA should be determined using samples from treatment-naïve subjects [37]. Bioanalytical laboratories establish the cut-point for antibody assessment if 1-10% of the tested treatment-naive samples are false-positive [31].

Detection of binding or neutralizing antibodies before and after drug injection is required to understand the immunogenicity, safety, and efficacy of gene therapy products based on AAV. It would be beneficial to have standard protocols in order to compare the data obtained from different studies of the immunogenicity of AAV vectors.

As Table 1 shows, ELISA and *in vitro* neutralization tests are most used in clinical trials to determine anti-AAV Ab titers. Although some studies show that binding and neutralizing AAV-specific IgGs correlate well [11], measuring NAbs is thought to be more desirable. Currently used assays for detecting anti-AAV NAbs include *in vitro* assays, *in vivo* models, and ex situ assays (Figure 1). *In vitro* assays are suitable for screening assays due to their simplicity and quickness. However, in scientific areas where more precise characteristics of a vector are required, *in vivo* models and ex situ assays may be used to obtain more information about the vector of interest.

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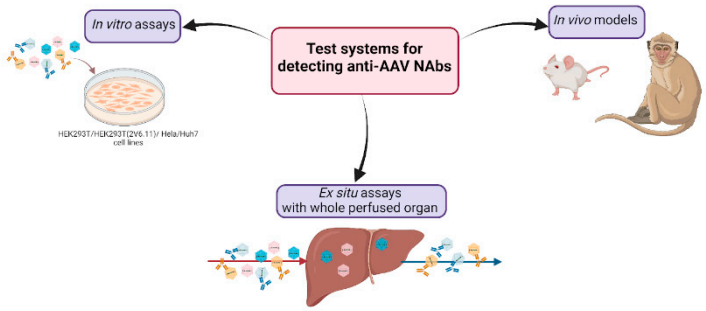

Figure 1. The present test systems for detecting anti-AAV Nabs.

We discuss below the advantages and troubles of the current assays used for evaluating serum NAbs to AAV.

4. *In Vitro* Neutralization Assay

The NAb titer to AAV is one of the most important criteria used to exclude patients for clinical trials and gene therapy treatment. Most clinical trials use *in vitro* assays to test NAb levels in patients' sera, because they are easy to set up and give consistent results [38].

The choice of target cell line is one of the important factors having influence on the results of the *in vitro* virus-neutralization assay (VNA). For SARS coronaviruses, the key cell receptor (ACE-2) is well-characterized [39]. Since the beginning of the COVID-19 pandemic, it has been relatively quickly shown that ACE-2 is a key cell receptor for the novel SARS-CoV-2 virus [40]. It enabled the rapid development and validation of a good *in vitro* model to detect anti-SARS-CoV-2 NAbs. Engineered cells with stable expression of ACE-2 are target cells in this assay [41]. Then, expression of TMPRSS was added to target cell lines [42] and now widely used in scientific studies and clinical trials.

Unlike SARS-CoV-2, there are no well-defined key cell receptors that are responsible for AAV transduction to the cells. Thus, glycan receptors are suggested to be low-specificity attachment factors, but they are not 'primary receptors' [43]. The potential key role of AAVR is also discussive [44]. Because of it, different cell lines are used in *in vitro* neutralization assays of AAVs. HEK293, Hela, COS-7, GM16095, and Huh7 cell lines are most used to determine the level of anti-AAV NAbs *in vitro* today, although transduction efficiencies of these cell lines vary by up to 5 orders of magnitude among different AAV serotypes [45,46]. Low transduction efficiency of several serotypes makes it impossible to determine NAb titers, due to the limit of detection. Higher multiplicity of infections (MOIs) can be used to overcome this problem, but it leads to underestimating NAb titers [38]. Moreover, lower MOIs in terms of *in vitro* VNAs enable cost reduction of the experiment because they require a lower number of AAV. As a result, more permissive cell lines suitable for transduction by most serotypes are highly desirable.

To improve transduction efficiency, target cells may be infected with adenovirus 5 before the neutralization assay [4,47,48]. 2V6.11, a cell line obtained from HEK293, overexpresses the adenovirus gene, E4 ORF, under the control of the ecdysone-inducible promoter. It was shown [19] that AAV6, 8, and 9 transduce this cell line more effectively than Huh7, HEK293, and HeLa, respectively. Hoi Yee Chow et al. established a Hela cell line with stable overexpression of AAVR and showed increased sensitivity of NAbs detection for different AAV serotypes [46].

The choice of cell line for *in vitro* neutralization assays is not the only important choice. Typically, VNAs consist of 2 steps: incubating viral particles with diluted serum and adding this mixture to target cells. Choosing the optimal dose of viral vector is pivotal when the goal is to compare neutralization titers against different serotypes. 3 VNT methods, and their limitations, are described below. Ultimately, good principles have been proposed. These principles are applied to VNA in general, in which other viruses are used.

The first and most wide-spread approach is to use a similar number of viral particles (measured by viral genomes, for example). The first limitation of this approach is that it does not consider the

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number of full and empty capsids. Empty particles compete with full AAV particles for binding to serum NAbs [47] and reduce the number of NAbs, blocking functional (full) AAVs. As a result, a large quantity of full AAVs can transduce target cells and express the reporter transgene, which will influence titration curves and neutralization titers.

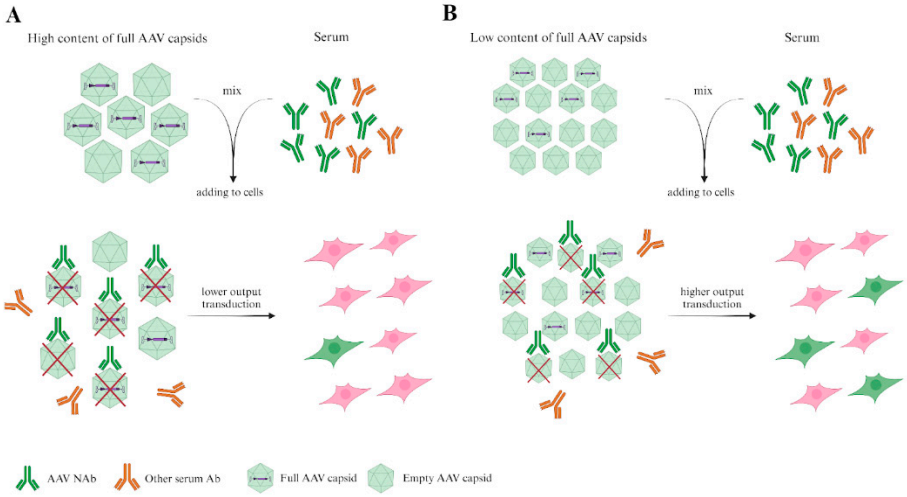

Figure 2. A figure illustrating the importance of considering the number of full and empty capsids in neutralization tests. Serotype A and serotype B are taken in equal numbers of viral particles measured by viral genomes. However, the serotype B sample contains a lower percentage of full capsids than serotype A. AAV NAbs bind not only full capsids, but also empty capsids, so the number of cells transduced by serotype B is higher.

For this reason, we propose that quality control tests of virus particles (VPs) are required. Tests of the ratio of full/empty capsids and the presence of VP aggregates may be appropriate. Taking viruses with a similar ratio of full/empty capsids is a good practice.

The second approach is to use the number of viral particles (VPs) that transduce a similar percentage of target cells [49]. This approach is limited by the varying ability of different serotypes to infect cells. Thus, in order to achieve the same level of transduction for different serotypes, it is necessary to select a number of VPs, which, in some cases, may differ by an order of magnitude. It is very important for chimeric AAV, which are often purposefully constructed to improve the abilities of transduction target cells [50–52]. Sera samples are traditionally serially 2-fold diluted and mixed with the chosen viral dosage. However, if the chosen dosages of viruses are dramatically distinguished, the ratio of serum antibodies and viral particles will be dramatically different. The lower number of VPs will require a lower number of NAbs to be neutralized, which will also have an influence on neutralization titers against different serotypes.

Thus, our recommendation is to use an equal ratio between the number of VPs and the volume of serum samples. The limitation that has affected both of the above approaches is the differing transduction levels of AAV serotypes. In our opinion, this limitation is the most difficult to overcome. If an equal number of AAV is used in an experiment for different serotypes, the transduction level may vary from 0 to 100% for different serotypes. Low transduction levels (about 10%) increase the significance of mistakes related to measurement, which will have an influence on titration curves and neutralization titers. If the number of transduced cells is close to 100%, it is not always clear which number of AAVs transduce cells in abundance. The appropriate transduction level for different serotypes may not always be achieved. Alternatively, if transduction level is used as a metric, then the same transduction level may be achieved by vastly different levels of VPs, with variances as high as in degrees of magnitude.

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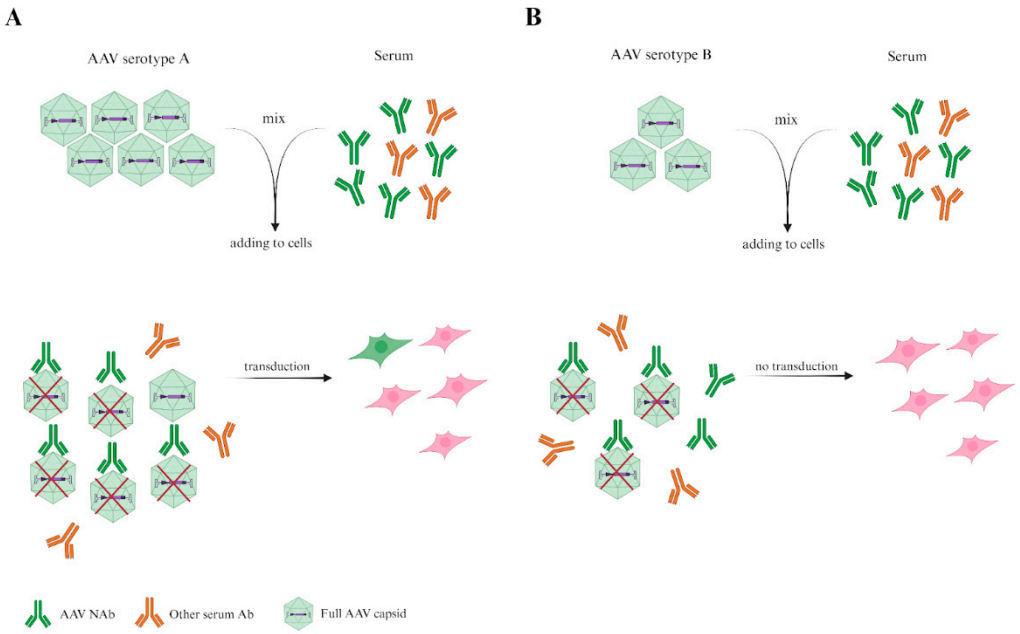

Figure 3. A figure illustrating the importance of the variance in the MOIs of AAV serotypes. Chosen doses of serotype A and serotype B transduce an equal number of target cells in the absence of serum antibodies (not shown in figure). However, in the presence of equal volume/number of specific AAV antibodies from serum, the levels of transduced target cells are different, which reflects the lower obtained viral dosage of serotype A, rather than the difference in antigenic characteristics between the two serotypes.

As previously discussed, incubation with an equal ratio of VPs and serum volume is needed. Thus, we suggest incubating VPs with serum and transferring the volume of this mixture, which, in the absence of serum, would transduce the necessary percentage of target cells. However, following this recommendation becomes challenging when MOIs differ by more than three orders of magnitude.

Unfortunately, in a number of articles NAb titer assessment to AAV, the methods used for assessment are not described in sufficient detail. Studies use different MOIs [4], different genomic MOIs [53,54], and different genomic numbers of AAV [53] for different AAV serotypes.

5. Determination of Immune Response to AAVs in Animal Models

As discussed above, the most difficult limitation to overcome in *in vitro* assay is the different transduction efficiency of cell lines. It should be underlined that this limitation is only relevant when the goal is to compare NAb responses to different AAV serotypes. The usage of animal models may provide extra insights in studying NAb response to AAVs and expand the methods used to determine the level of these NAbs.

A study by Mingozzi et al. (2018) demonstrated that, despite promising outcomes in animal models, human trials of AAV gene therapy have encountered unexpected immune reactions [55]. These differences highlight a challenge in translating findings about immune responses to AAVs from animal models to humans [56].

Mice and non-human primates (NHPs) are the most widespread animal models that are used to study immune response to AAV. However, the composition and role of immune cells differ between humans and mice [57]. Mestas and Hughes (2004) highlighted that human immune systems contain unique immune cell subsets not found in mice (CXCR1, CD58 (LFA-3), CD40 on EC, etc.), which can affect the study of viral infections and the immune response to AAV vectors [57]. NHP models have demonstrated the potential for unexpected immune reactions, including the activation of memory T cells and antibody responses that were not fully predicted by rodent models [58]. Despite their

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advantages, NHPs are not without limitations [59]. Rhesus macaques display a higher number of certain immune cells, such as CD4+/CD8+ double-positive T cells, than humans [60]. They may react differently to AAV vectors compared to cynomolgus macaques, leading to variability in study outcomes [59,61,62]. Furthermore, in vaccine studies, while NHPs like macaques often develop strong NAb responses, their immune system doesn't always mimic human long-term immunity or antibody decay rates, as seen in COVID-19 vaccine research [63]. Thus, there is a need to develop more appropriate systems to test human or human-like immune responses to AAVs.

Humanized mouse models would be valuable in studying the immune response to AAVs. These models aim to address the limitations of traditional animal models, offering more accurate predictions of human immune responses. Studies using these mice have shown that, while humanized models can replicate some aspects of the human immune response, such as T cell activation and antibody production, they may not fully recapitulate the complexity of human immune reactions to viral infections, particularly those involving regulatory T cells and innate immune cells like plasmacytoid dendritic cells (pDCs) [64].

The breakthrough mouse model with a complete, functional human immune system and human-like gut microbiome was created by the Paolo Casali group [65]. The most interesting result demonstrated in this model, in terms of detection of human humoral response, is that these (truly humanized) THX mice showed mature neutralizing antibody responses to Salmonella Typhimurium and SARS-CoV-2 virus after vaccination with Salmonella flagellin and the Pfizer COVID-19 mRNA vaccine, respectively. It would be desirable to test NAb formation that will reflect the human humoral response in these mice to other viruses, including AAVs.

Knock-in mice engineered to express human immune components offer a controlled system for studying, including AAV interactions. For instance, different studies used knock-in mice with human immunoglobulin loci that provided further insight into human-specific immune responses [66–68].

To summarize, the previously described animal models reflect human immune responses and develop the humoral response to AAV after vector injection to varying extents. However, induced humoral response to AAV is usually measured by *in vitro* assays, the limitations of which were also discussed above. Next, we describe animal models that allow the measurement of human-like NAb responses, fully avoiding the *in vitro* neutralization tests.

This approach involves inducing a human-like humoral immune system in animals, followed by AAV injection. The effectiveness of vector neutralization is compared to the same line of mice that weren't humanized.

In a study by Lan Sun et al. [69], researchers generated a mouse model of passive immunity to quantitatively assess anti-AAV8 NAb titers. C57BL/6 mice were first injected with rhesus macaque sera followed by scAAV2/8.CB.hAAT. The level of anti-hAAT Abs was measured by ELISA and inversely corresponded, according to the authors' study design, to the level of anti-AAV NAbs. This study showed that this *in vivo* NAb assay was more sensitive than an *in vitro* NAb assay; serum samples with low titers <1:5 NAbs were detected as positive to the anti-AAV8 response only by *in vivo* assay. Lili Wang et al. introduced this approach, but they transferred monkey sera to C57BL/6 mice prior to injection of AAVs with cFIX as a [70]. cFIX expression levels in mouse plasma were indicators of AAV neutralization.

Thus, in the first example, the level of NAbs to AAV was evaluated relative to the anti-transgene antibody level, whereas in the second example, it was relative to transgene expression. The level of viral DNA and/or transgene RNA may be included in the study design when this approach is used.

To summarize, animal models may be used in two ways. The first way is to use animals with a modified immune system or animals with an immune system close to human, inject AAV and measure NAbs in *in vitro* tests. In other words, this method may be used without the necessity for *in vitro* neutralization tests, which expands the possibilities when it comes to the detection of AAV NAbs.

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6. *Ex Situ* Assays with Whole Perfused Explants

The whole human explant offers another valuable perspective for evaluating the level of NAbs to AAVs. In this context, Marti Cabanes-Creus et al. (2024) introduced an *ex situ* system of normothermic human liver explants. One of them was perfused with human blood in the presence of anti-AAV NAbs that contained an AAV library [71]. The other liver explant was perfused with the AAV library only. The level of AAV neutralization corresponded to differences between two explants in the level of viral DNA and transgene cDNA, as determined by NGS. This method did not require *in vitro* neutralization assays.

This model is characterized by the ability to preserve the structure of human tissue, deliver the drug through a natural network of capillaries, and observe the neutralization of AAV in the presence of the resident macrophages, Kupfer cells. This allows plasma to be passed through the perfusion system under conditions close to physiological ones. The authors showed that, in the presence of human plasma containing anti-AAV NAbs, vectors derived from AAV2/AAV3b were extensively neutralized, unlike AAV8-derived variants. The authors noticed that AAV were rapidly cleared from perfusate if the explant contained NAb to the serotype. This may have been mediated by Kuppfer cells and could not be represented *in vitro*.

In general, the use of *ex situ* normothermal perfusion of human organs opens up prospects for studying the neutralization of AAVs in an environment containing different tissues and cell types, including the resident immune cells of the organ. Besides the liver, models of isolated hearts perfused with AAV already exist [72,73], and may potentially be used for detecting neutralizing antibody activity if human plasma is added to the perfusing solution.

Ex situ perfusion of human organs with NAbs and AAVs has several limitations. The availability of donor material is both generally limited, then further restricted by exclusion criteria for transplantation. The maintenance of the explants in a viable state for several days is also a difficult methodological task. Upon connection to the perfusion unit, donor organs may undergo reperfusion damage and fragmentation, compromising the capillary structure and, subsequently, the virus' distribution pathway. It is shown that, after six days of perfusion, markers of necrosis in the liver explant increased and organ functionality declined [74]. Moreover, the normothermic perfusion explant model is limited by the absence of circulating immune cells, which may potentially be involved in AAV response. The advantages of this model include that it does not require *in vitro* neutralization tests and partially reflects the interaction of AAVs with cells with immune function as it occurs in the whole organism.

7. Antigenic Map of AAVs Created on Published Data

The surface capsid proteins of different variants (strains, serotypes, isolates) of the same virus always have a certain homology in sequence and structure [43,75,76]. Such homology, which is closely linked with evolutionary relationships, may result in similarity of the physicochemical properties of the protein surface. This leads to cross-reactivity, when antibodies formed by the host in response to one variant of the virus neutralize another variant.

Despite several attempts to predict cross-reactivity *in silico* [77–79], the gold standard of antigenic similarity estimation is mass assessment of neutralizing properties of diverse serum samples. The serum samples can be taken from model animals immunized with a specific variant. However, as previously discussed, animals can develop similar, but not identical humoral responses to humans, which leads to unpredictable deleterious immune responses in AAV-based GTs clinical trials. Sera from conditionally healthy (not infected) individuals or who have been infected by a known virus serotype are preferable [80], but in the case of AAVs, this is not achievable. Titer neutralization data obtained from individuals who have been vaccinated with an AAV GT drug based on a known serotype provide sufficient data about the formation of cross-reactive antibodies.

Animal or human serum neutralization capacity is usually tested *in vitro*, the advantages and limitations of which were discussed above. If these limitations are overcome or compromises are found, the reliable comparison of humoral responses to different AAV serotypes becomes possible.

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It is highly preferred in studies in which, for example, chimeric AAVs are developed with the purpose of evasion of pre-existing humoral responses [45,81–83].

Over the past decades, many different studies have been conducted that have assessed the prevalence of neutralizing antibodies against various AAV serotypes in diverse human populations [4,7,84]. Such investigations not only provide critical information about the percentage of seropositive individuals (and hence the potential for clinical use of a particular virus variant), but also allow the assessment of antigenic similarity between different AAV serotypes. However, this data is often segmental and difficult to visualize and analyze.

Previously, this problem appeared when cross-reactive immune response to influenza virus strains or SARS-CoV-2 variants and was solved by the antigenic cartography approach developed by Dereck [85–90]. This approach visualizes a large dataset of titers for several serotypes on a map. The closer the serotypes from each other are located on the antigenic maps (represented by areas), the more antigenically similar they are. Sera from various patients, represented by circles, are closer to the variants that they better neutralize. One grid line on the map corresponds to a twofold dilution in the virus neutralization assay and is called an "antigenic unit".

Here, we collected data from three publications in which neutralization titers of sera were performed [91,92]. The description of cohorts and AAV serotypes for which neutralization titers are performed in each of these studies is summarized in Table 2.

Table 2. Summary of data used to construct antigenic map.

Group	Cohort Description	*In Vitro* Neutralization Test Features	Reference	AAV Serotypes Used								
				AAV1	AAV2	AAV2.5	AAV3B	AAV5	AAV6	AAV8	AAV9	AAVrh10
1	Osteoarthritis patient population	7.5 × 10^6 transducing units of AAV-GFP + 56 µL diluted synovial fluid dilution, 5 × 10^4 HIG-82 cells/well, GFP expression analyzed by FACS. The Nab titer is given as the dilution of synovial fluid required to obtain 50% inhibition of transduction by AAV.GFP, as compared to cells incubated with AAV.GFP alone.	Abdul T. Y. et al. 2023		☑	☑		☑				
2	Normal human donors	10^9 AAV-LacZ vg/well + 2-fold serial diluted serum samples (initial dilution, 1:20), 10^5 Huh7 cells/well, luciferase activity was measured by microplate luminometer. The NAb titer was reported as the highest serum dilution that inhibited AAV transduction by 50%, compared with the mouse serum control.	Giles A. R. et 2020		☑		☑			☑	☑	☑
3	Healthy and hemophilia B patient populations	70 ul of virus + serial diluted serum samples (initial dilution, 1:20), HEK293T cells, luciferase activity was measured by microplate luminometer. NAB titer (IC50) is the dilution at which antibodies inhibit Hek293T cell transduction with AAV-LUC by 50%.	Majowicz A. et al. 2019	☑	☑			☑	☑	☑		
4	Healthy and cystic fibrosis patient populations	10^8 AAV-AP vg/well + 100 ul diluted serum samples (initial dilution, 1:20), HTX cells, AP-positive focusforming units were measured. The highest dilution of serum that inhibited AAV transduction by 50% or more compared with the untreated vector was defined as the neutralizing titer.	Halbert C. L. et al 2006		☑			☑	☑			

Titer data was analyzed using the Racmacs R package [93] and plotted on an antigenic map (Figure 4). Areas consider experimental and statistical uncertainty of their location determined by both pairwise similarity (ability to neutralize the same antigens) and proximity to related antigens.

The map, in general, represents some expected patterns. For example, AAV2 is located in the center of the sera samples cloud as the variant with the most common neutralization in the human population. AAV5 and AAV2 are distant in the antigenic map. It is known that these two variants have low sequence homology [94]. Here, we show that they may also be antigenically distinct.

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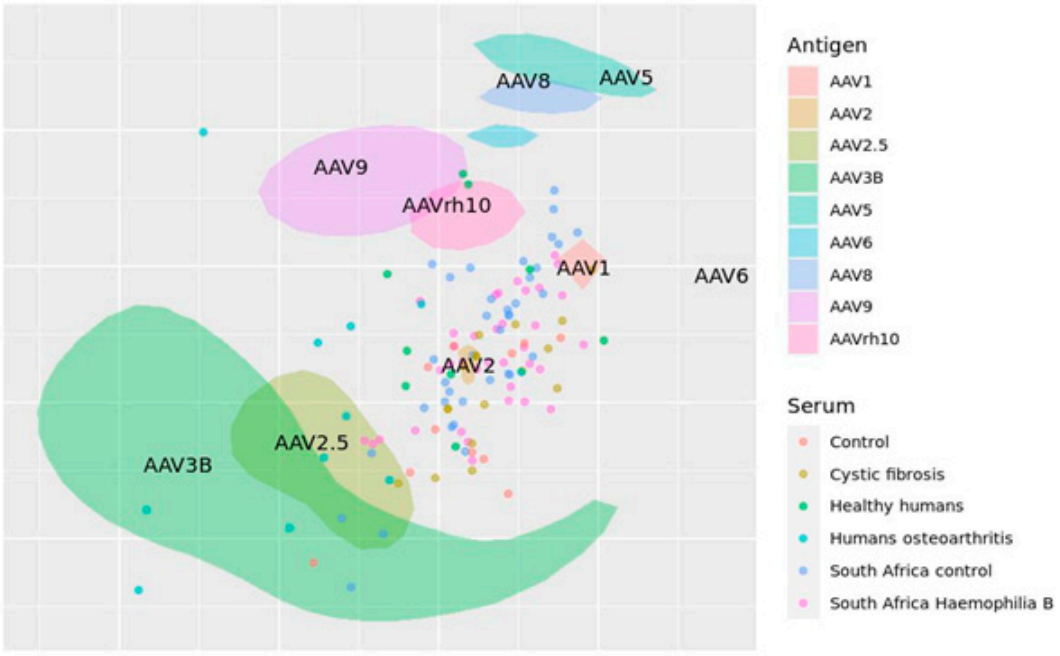

Figure 4. Antigenic map of AAVs constructed on published titer neutralization data. AAV serotypes are represented by areas, sera by circles. Areas consider experimental and statistical uncertainty of their location. One grid line corresponds to one antigenic unit.

It should be noted that the represented antigenic map has several limitations. Firstlyl, the used titers were obtained in different conditions in different laboratories. The number of studies we included in our analysis is low due to the limited availability of the raw data of neutralization titers in publications. In a study by Temilola Abdul et al, individuals with autoimmune disease (osteoarthritis) were included, although the humoral response may differ from healthy donors [92].

Taken together, for robust and reliable determination of the antigenic relationship between AAV serotypes, a systematic study is needed. This study must include many AAV variants (ideally, all variants of clinical importance) and a large homogeneous cohort of healthy individuals that are tested simultaneously. The limitations of neutralization assays should be reduced to a minimum with the recommendations we previously discussed. Such a study would provide valuable information for subsequent capsid design to evade neutralization by pre-existing antibodies.

8. Prospects and Future Directions

Pre-existing humoral immune response to AAV is a well-known limitation of GT application. The measurement AAV NAb titers and the determination of the threshold level, acceptable in GT, remain discussive. Here, we have shown the differences in antibody titers that are exclusion criteria for GTs. They may be explained by the absence of standardization of the assays used for determination of specific anti-AAV Abs. We focused on the details of these assays in the context of AAV, and reviewed alternative approaches to NAbs titer assessment, aside from the most popular *in vitro* assays. A more precise description of methods used in anti-AAV NAbs titer assessment and following good practice recommendations that we introduced here are necessary to increase the relevance and homogeneity of studies. The reliable comparison of titers to different AAV serotypes could help researchers design more effective vectors that avoid pre-existing immunity. The antigenic mapping approach is suitable for visualizing antigenic relationships between AAV serotypes. However, more reliable titer data are required to construct a more precise antigenic map.

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