Molecular Therapy
Methods & Clinical Development
Review

Preexisting antibody assays for gene therapy: Considerations on patient selection cutoffs and companion diagnostic requirements

Manuela Braun,[1] Claudia Lange,[1] Philipp Schatz,[1] Brian Long,[2] Johannes Stanta,[3] Boris Gorovits,[4] Edit Tarcsa,[5] Vibha Jawa,[6] Tong-Yuan Yang,[7] Wibke Lembke,[8] Nicole Miller,[9] Fraser McBlane,[10] Louis Christodoulou,[11] Daisy Yuill,[12] and Mark Milton[13]

[1]Bayer AG, Pharmaceuticals R&D, 13342 Berlin, Germany; [2]BioMarin Pharmaceutical Inc, Novato, CA, USA; [3]Celerion Switzerland AG, 8320 Fehraltorf, Switzerland; [4]Sana Biotechnology, 100 Technology Square, Cambridge, MA 02139, USA; [5]Abbvie Bioresearch Center, Worcester, MA 01605, USA; [6]Bristol Myers Squibb, Lawrence Township, NJ 08648, USA; [7]Janssen R&D LLC, Spring House, PA 19477, USA; [8]Integrated Biologix GmbH, 4051 Basel, Switzerland; [9]Ultragenyx Pharmaceutical Inc, Novato, CA 94949, USA; [10]Novartis, Basel, Switzerland; [11]UCB Pharma, 216 Bath Road, SL1 4EN Slough, UK; [12]AstraZeneca, 1 Francis Crick Avenue, CB2 0AA Cambridge, UK; [13]Lake Boon Pharmaceutical Consulting, LLC, Hudson, MA 01749, USA

Recombinant adeno-associated virus (AAV) vectors are the leading delivery vehicle used for *in vivo* gene therapies. Anti-AAV antibodies (AAV Abs) can interact with the viral capsid component of an AAV-based gene therapy (GT). Therefore, patients with preexisting AAV Abs (seropositive patients) are often excluded from GT trials to prevent treatment of patients who are unlikely to benefit[1] or may have a higher risk for adverse events outweighing treatment benefits. On the contrary, unnecessary exclusion of patients with high unmet medical need should be avoided. Instead, a risk-benefit assessment that weighs the potential risks due to seropositivity vs. severity of disease and available treatment options, should drive the decision if patient selection is required. Assays for patient selection must be validated according to their intended use following national regulations/standards for diagnostic assays in appropriate laboratories. In this review, we summarize the current process of patient selection, including assay cutoff criteria and related assay validation approaches. We further provide considerations on regulatory requirements for the development of *in vitro* diagnostic tests supporting market authorization of a corresponding GT.

INTRODUCTION

Recombinant adeno-associated viruses (AAVs) are the primary vectors for *in vivo* gene therapy (GT) delivery.[2] There is a high prevalence ($\leq$95%)[3–7] of antibodies against the AAV capsid due to prior exposure to wild-type AAVs (*wt*AAVs), which can cross-react with recombinant AAV capsids in GTs. These preexisting or treatment-induced AAV Abs can potentially impact treatment efficacy and cause adverse events. However, no correlation has been shown between AAV Ab levels and adverse events in preclinical[8] and clinical GT studies[8–10] (Table 1). Adverse events typically occur within the first 3 months after dosing[11–14] while treatment-induced AAV Abs emerge within 1–2 weeks after GT administration.[11,15] Nevertheless, patients with preexisting AAV Abs are often excluded from clinical trials. In such cases, the AAV Ab assay used for treatment decisions must adhere to national diagnostic standards and guidelines. Depending on the clinical development strategy and *in vitro* diagnostic (IVD) classification, the development of a complementary or companion diagnostic (CDx) may be required for commercialization of the GT.

In this article, we focus on assessing preexisting AAV Abs for subject selection across all GT development phases and post-launch. We provide an overview of approved AAV-based GTs and their related IVDs. We explore critical parameters for developing an assay strategy and describe various IVD types and their context of use (COU). In addition, we discuss the different assay formats used for AAV Ab assessment, including cutoff and titer determination approaches. We emphasize the distinctions between bioanalytical and diagnostic assays and laboratories, aiming to promote collaboration between these fields, facilitating method transfer as needed. Finally, we delineate the assay's journey, from early preclinical use to its role as a CDx. We also address regulatory requirements for assays used in patient selection, regulatory pathways for CDx market authorization, and the interaction between regulatory approval for the GT and the associated IVD.

TOTAL AND NEUTRALIZING AAV ABS, TITERS, AND SEROPREVALENCE

Total AAV Abs (AAV TAbs) can form immune complexes with the AAV capsid, redirecting the GT into complement and Fcγ receptor-bearing cells of the reticuloendothelial system, altering the viral vector's biodistribution *in vivo* and affecting treatment efficacy. This process further activates macrophages and dendritic cells, enhancing downstream adaptive immune responses.[16]

https://doi.org/10.1016/j.omtm.2024.101217.

Correspondence: Manuela Braun, Bayer AG, Pharmaceuticals R&D, 13342 Berlin, Germany.
E-mail: manuela.braun@bayer.com

Table 1. Impact of preexisting or treatment-induced immune response to the AAV capsid on safety and efficacy in preclinical and clinical studies

Phase; Species	GT; Dose; ROA	AAV Ab assay	Safety and efficacy observations in subjects with measured preexisting AAV Ab titers shown in bold	Immune response potentially triggering the adverse effects	Reference
Preclinical; NHP	AAV8-GFP; 3×10^{12} vg/kg; intravenous	TI assay, HuH-7, AAV8-LacZ, 50% inhibition	**preexisting TI titers ≥5:** reduced vector copy number and GFP expression in liver; increased vector copy number in spleen; higher treatment-induced AAV Ab titer	immune complex formation with AAV capsid	Wang et al.[90]
Preclinical; NHP (n = 14)	AAV5-*wt*hFIX (AMT-060); 5×10^{11}, 5×10^{12}, 2.5×10^{13}, 9.3×10^{13} vg/kg; intravenous	TI assay, HEK293T, AAV5-Luc, 50% inhibition	**preexisting TI titers between 57 and 1,030:** no impact on transduction or transgene expression; no liver toxicity or T cell response against the capsid	preexisting antibodies not associated with any adverse observations	Majowicz et al.[30]
Clinical (n = 10)	AAV5-*wt*hFIX (AMT-060); 5×10^{12}, 2×10^{13} vg/kg; intravenous	TI assay, HEK293T, 50% inhibition indirect TAb assay measuring IgG or IgM, MRD 1:50	**preexisting TI titers ≤340 (TAb IgG titers ≤256):** transient ALT elevation; titer 340 associated with highest FIX expression after low dose administration	preexisting antibodies and transient T cell response to AAV capsid not associated with any adverse observations	Hemgenix label; Pipe et al.[31]; Majowicz et al.[30]; Thornburg[29]
Clinical	AAV5-hFIXco-Padua (AMT-061, Hemgenix); 2×10^{13} vg/kg; Intravenous	TI assay, HEK293T, AAV5-Luc, 50% inhibition	Asymptomatic mild elevation of ALT/AST **preexisting TI titers ≤678:** hemostatic protection; comparable FIX expression in seropositive and seronegative patients **preexisting TI titer of 3,212:** no hemostatic protection; no FIX expression	injury of transduced hepatocytes; immune complex formation with AAV capsid	Thornburg[29]; Pipe et al.[31]; Hemgenix label
Clinical (n = 8)	AAV2-hFIX; 2×10^{11} to 1.8×10^{12} vg/kg; intramuscular	TI assay, AAV2-LacZ, 50% inhibition	**preexisting TI titers ≤1,000:** no impact on gene transfer and transgene expression	preexisting antibodies not associated with any adverse observations	Manno et al.[91]
Clinical (n = 7)	rAVV2-hFIX; 8×10^{10}, 4×10^{11}, 2×10^{12} vg/kg; portal vein infusion	TI assay, AAV2-LacZ, 50% inhibition	**TI titer of 2:** no adverse observations **TI titer of 17:** reduced FIX expression; transaminitis	cytotoxic T cell activity on transduced cells; immune complex formation with AAV capsid	Manno et al.[74]
Clinical	AAV9-SMN1 (Zolgensma); intravenous	indirect TAb assay measuring IgG, statistical cut point	**preexisting TAb titer ≤50:** transient elevation of ALT, AST, and/or bilirubin concentrations (in most cases mild); TMA	increase of treatment-induced AAV9 TAbs co-incidental with liver enzyme elevation; TMA associated with complement activation (alternate pathway) and patient disease-related factors	Chand et al.[11]; Chand et al.[13]
Clinical	rAAVrh74.MHCK7. micro-dystrophin (SRP-9001); 2×10^{14} vg/kg; peripheral limb vein	indirect TAb assay, no statistical cut point, MRD 1:25 used as cutoff	**TAb titer ≤400:** elevated transaminase and transient elevation of gamma-glutamyltransferase levels	transient T cell response not associated with liver enzyme elevation	Mendell et al.[92]; Horton et al.[9]; Li and Song[93]
Clinical	rAAV9-micro-dystrophin (SGT-001); 5×10^{13}, 2×10^{14} vg/kg; intravenous	no information available	**no AAV9 Abs:** thrombocytopenia; reduced platelet count; liver dysfunction; kidney injury	complement activation	Dreghici et al.[94]; Horton et al.[9]; Li and Song[93]

(*Continued on next page*)

Table 1. *Continued*

Phase; Species	GT; Dose; ROA	AAV Ab assay	Safety and efficacy observations in subjects with measured preexisting AAV Ab titers shown in bold	Immune response potentially triggering the adverse effects	Reference
Clinical	rAAV9-mini-dystrophin (PF-06939926); 1×10^{14}, 3×10^{14} vg/kg; intravenous	TI assay	**no AAV9 Abs:** acute kidney injury involving aHUS-like complement activation	complement activation	Horton et al.[9]; Li and Song[93]

AAV Ab, anti-AAV antibody; aHUS, atypical hemolytic uremic syndrome; ALT, alanine aminotransferase; AST, aspartate transferase; DMD, Duchenne muscular dystrophy; GFP, green fluorescent protein; hFIX, human factor IX; LacZ, lacZ gene encoding the enzyme β-galactosidase; Luc, luciferase; MRD, minimum required dilution; NHP, non-human primates; ROA, route of administration; SMN1, survival motor neuron 1; Tab, total antibody; TI, transduction inhibition; TMA, thrombotic microangiopathy; vg/kg, viral genomes/kg.

Neutralizing AAV Abs (AAV NAbs), a subset of AAV TAbs, bind to epitopes on the AAV capsid surface crucial for receptor binding, thereby preventing cell entry and reducing transduction efficiency and treatment efficacy. Some AAV NAbs inhibit capsid processing post cell entry or the transfer of genetic material into the target cell nucleus.[17]

Immune responses are typically polyclonal and consist of various immunoglobulin isotypes, such as IgG1, IgG2, IgG4, and IgM, with different affinities and specificities for various antigen epitopes. Therefore, measuring antibody concentration (e.g., mg/L) does not adequately reflect the binding or neutralizing capacity. Instead, a titer is reported as the reciprocal of the highest sample dilution (e.g., dilution 1:128 = titer 128) that generates the last measurement signal before a predefined cutoff is reached.

Seroprevalence indicates the percentage of individuals with AAV Abs. AAV Ab titers and seroprevalence vary widely for different serotypes and are influenced by age and geographical location, presumably reflecting differences in environmental factors including regional waves of infections with *wt*AAVs, after which previously negative individuals seroconvert.[3–7,18–20] Seroprevalence in newborns can be high due to maternal antibody transfer, which declines during the first year of life, often leading to a complete loss of AAV Abs (seroreversion). As individuals encounter *wt*AAVs in the environment, seroprevalence increases with age.[4,21]

Ideally, seroprevalence should be similar in age- and geography-matched patients and healthy individuals, assuming the disease or treatment does not affect the immune system.[21] Reliable seroprevalence data require a statistically significant sample size based on expected seroprevalence. Published data vary due to diverse assay formats and conditions.[22–25] Seroprevalence is expressed either as the percentage of patients testing positive in a particular assay[5] or as the percentage of patients with an AAV Ab titer above a specific value.[26]

APPROVED AAV-MEDIATED GENE THERAPIES AND RELATED CDx OR LABORATORY-DEVELOPED TESTS

In this section we give an overview of the approved GTs and available IVDs detecting antibodies to their AAV capsids.

In December 2023, six *in vivo* GTs were approved for various indications and, notably, patient testing for preexisting AAV Abs varies across these GTs (Table 2).

Luxturna is approved in the USA and the EU for retinal pigment epithelium specific 65 kDa protein-associated inherited retinal dystrophy via subretinal injection and does not mandate preexisting AAV2 Ab testing in the GT label due to trial inclusivity (Table 2).[27] Regulatory guidance does not emphasize AAV Ab screening.[28]

Upstaza is approved in the EU for aromatic L-amino acid decarboxylase deficiency (Table 2). Safety and efficacy data for preexisting AAV2 NAb titers >20 are absent. However, the GT label does not specify testing for AAV2 Abs, presumably because they are considered less relevant for brain-local administration.

Hemgenix is approved for intravenous treatment of factor IX (FIX) deficiency in the USA, irrespective of preexisting AAV5 NAb status (Table 2). Initial trials screened for AAV5 NAbs, but retrospective analysis with a more sensitive assay, revealed that preexisting AAV5 NAbs (titers <340) did not impact treatment.[29,30] Seropositive patients were included in later trials confirming this outcome up to preexisting NAb titers $\leq$678, but one patient with a Nab titer of 3,212 had to restart FIX prophylaxis due to lack of transgene expression.[31]

Roctavian is approved for systemic treatment of severe FVIII deficiency in the USA and EU (Table 2). The GT labels mandate the absence of AAV5 Abs before treatment. An AAV5 DetectCDx Kit (Table 3) is available for this purpose and specified in the GT label in the USA. Although the AAV5 DetectCDx Kit is authorized for use in the EU since January 2022, the GT label only requests the use of an appropriately validated assay for AAV5 Ab testing.

Elevidys is approved in the USA for Duchenne muscular dystrophy (Table 2). Product label mentions that preexisting AAV Abs can impede transgene expression, and recommends patient selection based on AAVrh74 TAb titers but, currently, there is no FDA-authorized test for AAVrh74 TAbs, mainly due to accelerated approval of the GT.

Table 2. Overview on approved gene therapies, their patient selection criteria, and associated CDx

Name	Hemgenix	Roctavian	Zolgensma	Elevidys	Luxturna	Upstaza
INN	(Etranacogene Dezaparvovec)	(Valoctocogene Roxaparvovec)	(Onasemnogene Abeparvovec)	(Delandistrogene Moxeparvovec-rokl)	(Voretigene Neparvovec)	(Eladocagene Exuparvovec)
Serotype	AAV5	AAV5	AAV9	AAVrh74	AAV2	AAV2
transgene	hFIXco-Padua	BDD hFVIII	SMN1	mini dystrophin	RPE65	ADCC
ROA	intravenous infusion	intravenous infusion	intravenous infusion	intravenous infusion	subretinal injection	infused into putamen
Disease	Hem B	severe Hem A	SMA	DMD	IRD with RPE65 mutation	AADC deficiency
Patient age	adults	≥18 years	<2 years	4–5 years	pediatrics, adults	≥18 months
SOC	ERT	ERT	N/A	steroids	N/A	N/A
Approved in	USA, EU (conditional)	USA, EU	USA, EU, Japan	USA	USA, EU	EU
Patient selection[a] in pivotal trials	no patient selection	no AAV5 TAbs	TAb titer ≤50	AAVrh74 Ab titers <400	no patient selection[27]	AAV2 NAb titers ≤20
Patient selection after GT launch (product label)	no patient selection requested	select AAV5 Tab-negative patients using FDA-approved CDx (USA) or appropriately validated assay (EU)	AAV9 TAb testing recommended[32] but no patient selection requested; efficacy/safety not evaluated in patients with AAV9 TAb titers >50 (USA, EU); select AAV9 TAb-negative patients using approved MEBCDX AAV9 test (Japan)	select patients with AAVrh74 TAb titers <400; higher titers of AAV Abs may impede transgene expression; approved under accelerated approval	no patient selection requested	no patient selection requested; no safety/efficacy data for patients with AAV2 NAb titers >20
Companion diagnostic	no CDx	AAV5 DetectCDx Kit (USA); no CDx (EU)[b]	no CDx (USA, EU); MEBCDX AAV9 test (Japan)	currently no FDA-authorized test for AAVrh74 TAb detection available	no CDx[c]	no CDx

AADC, aromatic L-amino acid decarboxylase; CDx, companion diagnostic; DMD, Duchenne muscular dystrophy; ERT, enzyme replacement therapy; FVIII, factor VIII; FIX, factor IX; GFP, green fluorescent protein; Hem, hemophilia; IRD, inherited retinal disease; i.v., intravenous; Luc, luciferase; N/A, not available; RPE65, retinal pigment epithelium specific 65 kDa protein; SMA, spinal muscular atrophy; SMN1, survival motor neuron 1.

[a]Based on preexisting AAV Abs.

[b]Although AAV5 DetectCDx Kit test kit is Communauté Européenne (CE) marked and available in EU.

[c]FDA guidance on gene therapy for retinal disorders[28] does not mention patient selection based on AAV Abs.

Zolgensma treats spinal muscular atrophy and recommends baseline testing for AAV9 Tabs, although a prior exposure of the pediatric patients to AAV9 is uncommon (Table 2).[32] The GT labels mention absence of safety/efficacy data for preexisting AAV9 TAb titers >50 but no restrictions based on AAV9 TAb titers are mentioned in the USA and EU label, leaving the final decision to the clinician. Japan has more restrictive wording in the package insert and mandates patient selection using the approved MEBCDX AAV9 test (Table 3). In the USA and EU, no approved AAV9 Ab CDx exist. In the USA, two AAV9 Ab LDTs are approved by the state of New York, developed by Athena Diagnostics and Cellular Technology Limited (Table 3). An ELISA-based AAV9 TAb assay from Viroclinics Bioscience supports Zolgensma worldwide, excluding the USA and Japan. The assays used in Zolgensma clinical development by Viroclinics Biosciences and Cellular Technology Limited were aligned for various parameters,[32] but some assay details, such as the minimal required dilution (MRD), cut point, titer determination, positive control, and assay sensitivity remain undisclosed. Titers measured with Athena Diagnostics' AAV9 TAb LDT may not be directly comparable with those in Zolgensma clinical trials.

While there are six approved AAV-based gene therapies as of December 2023, only two CDx exist for these therapies, demonstrating that the necessity for a CDx varies based on approving regulatory authority, patient selection criteria in clinical studies, and the assessment of risks and benefits associated with GT. A lack of regulatory guidance has allowed some gene therapies in the EU to be approved without a CDx, but this may change with the new regulations. Additional GT authorizations are anticipated, some of which may require a CDx based on the test's utility and the treated patient population.

PREEXISTING AAV ABS AND THEIR IMPACT ON PROJECT FEASIBILITY AND NEED FOR PATIENT SELECTION

Preexisting AAV Abs may affect safety and efficacy of a GT treatment. Therefore, based on the overall risk-benefit associated with the treatment of seropositive patients, sponsors must decide if patient selection is necessary. In this section, we discuss various patient- and treatment-related factors relevant for the overall risk-benefit assessment and the impact of patient selection on project feasibility (Table 4).

Table 3. Overview on diagnostic anti-AAV9 and anti-AAV5 antibody tests

Serotype	Assay format	Analyte	Cut-off titer	Matrix	Test name	Certificate	Provider	Available
AAV9	ELISA (indirect, peroxidase)	TAb (IgG)	ND	serum	MEBCDX AAV9 test	CDx (single site)	MBL International	Japan
AAV9	ELISA	TAb (IgG)	50	serum		LDT (single site)	Cellular Technology Limited[a]	USA
AAV9	ELISA (indirect, peroxidase)	TAb (IgG)	50	serum		LDT	Viroclinics Biosciences BV[b]	Worldwide except for USA and Japan
AAV9	ELISA (N/D)	TAb (N/D)	25	serum		LDT (single site)	Athena Diagnostics Inc.	USA
AAV5	ELISA (bridging, ECL)	TAb (all Ig subtypes)	N/D qualitative assay	plasma, citrated	AAV5 DetectCDx^{M} Kit	CE marked (single site)	ARUP PharmaDX Clinical Laboratory	EU
AAV5	ELISA (bridging, ECL)	TAb (all Ig subtypes)	N/D qualitative assay	plasma, citrated	AAV5 DetectCDx Kit	LDT (single site)	ARUP PharmaDX Clinical Laboratory	USA

[a]Used in STR1VE-US and SPRINT clinical study with Zolgensma.
[b]Used in STR1VE-EU clinical study with Zolgensma. CDx, companion diagnostic; CE, Communauté Européenne; ECL, electrochemiluminescence; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; LDT, laboratory developed test; N/D, not disclosed; TAb, total antibody.

Medical need and available standard of care options and their impact on patient selection considerations

Medical need and available standard of care (SOC) treatment options on the one hand and the benefit and risks associated with the treatment of seropositive patients on the other hand affect the need for patient selection.[33,34] In this section, we discuss the overall risk for a patient associated with inappropriate patient selection (Table 4).

An inappropriately high cutoff for patient selection or a false-negative test result can lead to the treatment of seropositive patients who will probably not benefit from the treatment and may experience adverse events. This must be avoided as seroconversion currently prevents gene transfer in subsequent treatments and can also prevent alternative GT treatments due to cross-reactive AAV Abs. An inappropriately low cutoff for patient selection or a false-positive test result leads to unnecessary exclusion of patients. This is critical if no or no optimal SOC treatment is available, especially when the treatment benefit outweighs the risks associated with the treatment of seropositive patients. In such a case, patient selection may be unnecessary or even unethical and the sponsor should get the GT to patients as quickly as possible. If a severe disease is manageable with approved alternate SOC treatment, such as for hemophilia A or B, the risks associated with treatment of seropositive patients may outweigh the benefit on top of SOC making patient selection necessary.

Overall, sponsors must balance patient's needs with the risks posed to a seropositive patient when defining patient selection criteria for GT treatment and the assay used for patient selection must generate reliable data in a robust manner.

Route of administration and its impact on patient selection

In this section, we discuss various routes of administration (ROA) and their impact on the risks associated with treatment of seropositive patients (Table 4).

Systemic intravenous administration to transduce a peripheral tissue or a blood-tissue barrier protected tissue

Systemic intravenous administration is employed to transduce peripheral tissues (e.g., skeletal muscle and liver) or protected tissues (e.g., brain). It often necessitates higher per-patient doses and can lead to broader AAV distribution. Preexisting or treatment-induced AAV Abs may form immune complexes with the AAV capsid, potentially impacting treatment effectiveness and safety. For instance, Roctavian and Hemgenix use intravenous administration to transduce peripheral liver tissue. Meanwhile, Zolgensma employs this method to transduce protected motor neuron cells within the spinal cord and brain.

Overall, systemic administration involves higher per-patient doses, which may pose greater risks for seropositive patients when compared with local administration.

Local administration to a peripheral tissue for direct transduction of that tissue or for transduction of target cells in that tissue that are protected by a blood-tissue barrier

Local administration's systemic exposure depends on dose, AAV tropism, target tissues, and site of administration. Administering to peripheral tissues requires lower doses per patient, reducing safety risks compared with intravenous administration. Preexisting AAV Abs may have a smaller impact after local administration, but this depends on target tissue transduction time and the GT amount in relation to local AAV Abs. Administering a fixed dose to children of varying ages may result in higher systemic concentrations in those with lower body weight, potentially leading to more and larger immune complexes with AAV Abs than in children with higher body weight.

These considerations also apply when protected tissues such as peripheral nerves or the CNS are targeted by local administration to peripheral tissues.[35] Here, the impact of AAV tropism and the transduction efficiency is more significant. For instance,

Table 4. Some relevant factors to consider for risk-benefit assessment based on preexisting anti-AAV antibodies

	Impact on project feasibility		
Business risk	High	Medium	Low
Prevalence and titer of preexisting AAV Abs	high seroprevalence with high titers likely reduces the number of eligible patients in case of, e.g., systemic administration	high seroprevalence with low titers or low seroprevalence might potentially affect the number of eligible patients in case of, e.g., systemic administration	seroprevalence has a lower impact on the number of eligible patients in case of, e.g., local administrations, in particular to "immune privileged" tissues

	AAV Ab screening may be		
Patient-/treatment-related risk	required	considered	not necessary
Medical need (SOC and severity of disease)	disease manageable with approved	intermediate medical need,	high medical need,
	alternative SOC	no optimal SOC available	no (optimal) SOC available
	example: hemophilia A/B	example: Alzheimer's disease	examples: DMD, SMA
Route of administration (likelihood of GT interaction with preexisting antibodies)	systemic administration	local administration into target tissues	local administration to "immune privileged" tissues
	examples: hemophilia A/B, local administration to peripheral tissues (not "immune privileged")	examples: intracoronary or intramuscular administration	example: subretinal or into putamen administration

AAV Ab, anti-AAV antibody; SOC, standard of care; DMD, Duchenne muscular dystrophy; SMA, spinal muscular atrophy.

intramuscular administration of a neuron-tropic GT can result in extensive transduction of spinal cord, brainstem, and sensory ganglia cells due to motor and sensory neuron terminals residing in skeletal muscle.[35]

In summary, local administration to peripheral tissues can reduce risks associated with treating seropositive patients and is a minimally invasive alternative to local administration to blood-tissue barrier-protected tissues.

FDA guidance recommends considering exclusion of seropositive patients and exploring CDx development for both local and systemic administrations.[28,36–38]

Local administration to a blood-tissue barrier-protected target tissue

Even after local administration to protected target tissues (e.g., intrathecal, intracerebral, intravitreal, subretinal), the GT may enter the systemic circulation. The extent of systemic exposure depends on the targeted tissue, AAV tropism, administered dose, dosing procedure, and the patient's disease state, which can affect systemic exposure and the likelihood of observing systemic adverse events due to barrier function impairment.[28] As long as the blood-tissue barrier remains intact, it can prevent antibodies from reaching the protected tissue.[39] However, the strength of the barrier protecting a particular tissue varies based on its functional requirements.[40,41] For example, in mice, total AAV Ab levels in brain tissue were only 0.6% of systemic circulation levels.[39] Similarly, in humans with an intact blood-ocular barrier, AAV5 and AAV2 NAb titers in the vitreous were approximately 80- and 300-fold lower compared with corresponding serum titers.[41] Based on these limited published data, low or no preexisting AAV Ab levels are expected in blood-tissue barrier-protected compartments. Since local administration results in relatively high local GT concentrations, the impact of low preexisting AAV Abs on efficacy is unlikely.

However, in cases where broader systemic distribution is expected due to AAV serotype tropism or an impaired blood-tissue barrier (due to disease or administration procedure), the impact of preexisting AAV Abs on safety and efficacy becomes possible.[41] In such situations, exclusion of seropositive patients may be necessary, considering the risks associated with both central and peripheral GT distribution. AAV Ab assessment in the protected compartment, if accessible, should be considered, as local AAV Abs in areas such as cerebrospinal fluid are more relevant for assessing their impact on efficacy. Examples of local administration to blood-tissue barrier-protected compartments include Upstaza with intraparenchymal CNS delivery and Luxturna with subretinal injection.

Overall, local administration to blood-tissue barrier-protected compartments reduces potential risks associated with AAV Abs detected in serum/plasma and may eliminate the need for patient selection as long as the barrier remains intact.

Preexisting AAV Abs and their impact on project feasibility

Requiring patient selection can complicate and prolong GT development, increasing costs and potentially rendering the GT commercially unviable. To mitigate these challenges, we recommend determining the eligible patient population early in project development using the intended clinical trial assay (CTA). Depending on patient age, careful consideration of the treatment time window and retesting of seropositive patients can expand the eligible patient pool. For instance, seroprevalence in newborns generally decreases in the first few years of life, potentially reaching nearly 100% patient eligibility.[42] However, seroprevalence tends to increase with age, particularly during the first 20–30 years of life.[4,5] Therefore, it is essential to account

for seroreversion and seroconversion when defining the treatment window.

In conclusion, reliable patient eligibility data can only be obtained using the intended CTA, as seroprevalence is heavily influenced by the assay format and chosen cutoff. The testing strategy must consider seroreversion and seroconversion to determine treatment window and patient eligibility effectively.

VALIDATION REQUIREMENTS FOR DIAGNOSTIC ASSAYS ACCORDING TO CONTEXT OF USE

If patient selection is deemed necessary for the safe and effective use of their GT, the development of a CDx is required.

Different types and contexts of use of diagnostic assays

In this section, we provide an overview of diagnostic tests and their clinical COU, along with considerations for CDx development. Different types of diagnostic tests and their validation requirements are discussed:

IVDs are medical devices used for physician-requested testing of patient samples. These tests are typically conducted in clinical diagnostic laboratories, hospitals, or by commercial providers. They can be centralized in accredited/certified laboratories or distributed by IVD manufacturers to specific accredited/certified laboratories.

CTAs are designed to support subject selection or investigate potential correlations between test results and clinical outcomes, such as safety or efficacy observations.[34] CTAs are used for clinical trials but may become approved IVDs later, if necessary. Sponsors may consider using an available IVD as a CTA. However, if a CDx is expected to be required, sponsors should discuss the potential premarket approval (PMA) submission of the IVD as a CDx with the test provider early in the process.

A CDx is a medical device, often an IVD, that provides essential information for the safe and effective use of a corresponding drug or biological product.[33,43] The CDx information is included in the "intended use" section of the therapeutic product's label, and the drug and device are cross-referenced. Drug and device manufacturers need to limit their promotion to statements provided in the label. In the USA and Japan, the drug is typically approved only when the corresponding CDx is available. In Japan, treatment decisions can only be made based on patient testing with the Pharmaceuticals and Medical Devices Agency (PMDA)-approved CDx. In the EU and USA, physicians can use test results to select targeted therapies at their discretion, but medical laboratories need to comply with national requirements, such as CAP/CLIA (College of American Pathologists/Clinical Laboratory Improvement Amendments) in the USA or In-vitro Diagnostic Regulation (EU) 2017/746 (IVDR)[43] in the EU. Sponsors should consider that only a limited number of diagnostic laboratories are ISO13485 certified and can support analytical CDx development and regulatory submissions when selecting a partner.

A single-site CDx is a specific variant of a CDx developed and validated in an accredited/certified diagnostic laboratory at a single site to support a PMA or become IVDR compliant. It is not available as a distributed kit. Examples include F1CDx, BRCAnalysis, Guardant360, MEBCDX AAV9 test, and AAV5 DetectCDx Kit. When the CDx or the prototype CDx is not available during clinical development, an LDT or a fit-for-purpose validated CTA can be used.

Complementary diagnostics are tests that are not essential for the safe and effective use of a corresponding drug but provide meaningful information for individual patient risk-benefit evaluation and treatment decisions. Complementary IVD information is included in the clinical section of therapeutic product labeling, not in the intended use section.[44,45] Complementary diagnostics are currently approved primarily for PD-L1 testing.[46]

Class complimentary diagnostics can be used for multiple products within a therapeutic class.

Laboratory-developed tests (LDTs) or in-house tests are locally developed in specific laboratories and are not commercially available as distributable test kits. They can be either supplemental validated research use only tests or validated IVDs used outside the intended use statement from the package insert. LDTs are employed when a CDx or an IVD is not available at the time or site of a clinical trial.

In the EU, laboratories developing LDTs (in-house IVDs) must follow IVDR requirements and meet single health institute criteria outlined in Article 5(5) of IVDR.[43] Such laboratories must be established in the EU or Northern Ireland, focusing on patient care or public health promotion, with LDT testing limited to a non-industrial scale. Respective LDTs need to meet the relevant safety and performance requirements set out in Annex I of the IVDR.[43] Additional requirements will become effective at the end of the transitional provisions of IVDR (2024–2028).

In the USA, LDTs are regulated by the Centers for Medicare and Medicaid Services (CMS), with clinical diagnostic laboratories regulated at the federal level through CLIA. Federal CLIA regulatory authority and individual state authorities oversee laboratories, with New York State having particularly stringent requirements. A summary of the different types of diagnostic tests, with examples and their COU, is provided in Table S1. Different types of diagnostic tests serve various purposes in guiding patient treatment and should be carefully considered in the development and approval of GTs and their corresponding diagnostics. Regardless of whether the assay is an LDT, IVD (510(k) or CE marked), or CDx, it must provide reliable results over years or even decades. A rapid turnaround, ensuring availability of results within days for all patients globally, is crucial. The assay may be established in multiple diagnostic laboratories around the world in a decentralized manner if urgent treatment after diagnosis is critical or there is a high risk of seroconversion, especially in children. For centralized single-site assays, time from sampling to test result availability may be longer. Early consideration of long-term and local

reagent availability and shipping requirements (e.g., shipping temperature and duration, sample stability, ability to export/import samples) is recommended.

The level of diagnostic assay validation depends on the risks posed to patients being tested falsely negative or falsely positive, assay complexity, COU, and national regulations. Regional regulations, such as those in China regarding human genetic resources, may impact testing laboratory selection. The use of different assays during GT development phases is discussed further in assay journey from preclinical use to CDx.

Strategic considerations for CDx development

Patient selection and CDx development hinge on regional regulatory guidance and vary with the risk-benefit assessment due to differing seroprevalence and available SOC across countries. If the overall risk-benefit assessment is unfavorable, excluding seropositive patients becomes necessary. The responsibility lies with the sponsor to establish the GT's safety and efficacy through clinical trials and provide clinicians with the tools needed for treatment decisions in practice.

SELECTION AND VALIDATION OF ASSAYS FOR ASSESSMENT OF AAV AB

This section details current AAV Ab assay formats and approaches to define cutoffs for titer determination. Bioanalytical cut points, typically used for immunogenicity post-biologic drug treatment, have been applied to preexisting AAV Abs for GT patient selection.[47–49] We compare these with diagnostic test cutoffs—reference intervals (RIs)/reference limits and clinical decision limits (CDLs)—clarifying similarities, differences, and clinical relevance. The subsequent section delves into assay use, optimization, and development phases in both bioanalytical and diagnostic laboratories. A glossary provides definitions aligning terminologies from bioanalytical and diagnostic guidelines to enhance collaboration and facilitate assay transfer between both laboratories (Table S2).

Assay formats used for AAV Ab assessment

In this section, we highlight key features of prevalent assay formats for AAV Ab assessment as described comprehensively in published literature.[22–25,47–49]

The ligand binding assays identify all antibodies (AAV TAbs) that bind to the AAV, while the functional cell-based assays characterize AAV NAbs. Bridging ligand binding assays generally detect all immunoglobulin subtypes, while indirect assays may target IgG, IgG, and IgM, or nearly all subtypes (excluding IgE).[22] Bridging assays involve AAV-specific capture and detection steps, while indirect ligand binding assays may lead to higher background levels due to detection of antibodies that are not AAV specific, potentially reducing sensitivity or causing more false positives.[50] Cell-based transduction inhibition (TI) assays not only identify AAV NAbs but may also yield positive results for non-antibody factors hindering vector uptake, endosomal escape, capsid processing, genetic material transfer, or reporter gene expression.[17,23,24,47,51]

In summary, various assay formats exist for AAV Ab assessment, each with its specific characteristics, leading to potential false-positive or false-negative outcomes. Considering the assay properties and the impact of AAV TAbs and NAbs on safety and efficacy in GT treatments, TAb assays appear more suitable for assessment and potential patient selection. In addition, adherence to acceptance criteria in IVD guidelines, primarily designed for non-cellular *in vitro* assays, may complicate the validation of TI assays as IVDs or CDx.

Approaches to define cutoffs for determination of AAV Ab titers

There is a lack of specific guidelines for GT immunogenicity assessment, prompting sponsors to adopt diverse approaches for method validation and cutoff determination. Bioanalytical laboratories, experienced in antibody assessment for biologic drugs,[47–49] utilize FDA and EMA immunogenicity guidelines[50,52] and white papers[53–56] employing a tiered sample testing approach involving screening, confirmation, and titer determination. The screening step mirrors IVD cutoff determination, although these guidelines do not apply to diagnostic assays and notable differences exist in validation stringency. The further characterization steps described in these guidelines are valuable method characterization tools.

Bioanalytical laboratories establish a screening cut point for antibody assessment in study samples, aiming to validate a 5% or 1% false-positive rate by calculating the one-sided 95th or 99th percentile of 50 treatment-naive control samples (Figure S1).[54] This mirrors the determination of an upper reference limit[57] for IVDs, although IVDs undergo a more stringent validation process with 90% confidence, using a minimum of 120 qualified individual reference samples for cutoff determination. Furthermore, IVDs undergo a more extensive robustness assessment at a CDL applying more stringent acceptance criteria[58] compared with screening cut points. In GTs, selecting a suitable negative control population (= reference population) is crucial due to prior human exposure to *wt*AAVs. Patient AAV Ab titers, assessed during eligibility screening, typically range up to 10,000,[11] with seroprevalence rates varying from 10% to 95%.[1,3–7,19,21,59] Thus, screening an adequate number of individual samples, reflecting the population's variability, helps identify those without AAV Abs. A thorough characterization of positive test results including confirmation steps, standard in bioanalytical laboratories, helps distinguish true AAV Ab-positive samples from false positives. For cell-based TI assays, confirming the AAV specificity is crucial. While it is unclear if non-antibody factors affecting TI assays *in vitro* impact clinical outcomes,[60] general immunodepletion confirms that the positive result is due to antibodies, for which an impact on GT treatment has been described.[29–31,51] AAV-specific confirmation, involving excess AAV capsid- or AAV-specific antibody depletion using AAV-coated resins, adds value in both ligand binding and TI assays when compared with immunodepletion. While successful validation of confirmatory steps in TI assays has been described,[49] these techniques are presently underutilized. Post confirmation, laboratories determine titers to gauge binding or neutralizing capacity. For conventional biologics, titer values always refer to undiluted samples, incorporating all dilution steps for easier data interpretation.

An alternative AAV Ab titer determination method in TI assays is the non-statistical 50% inhibition approach,[25] often used in preclinical or research settings (Figure S2).

The cutoff approaches presented here are technical, indicating AAV Abs presence without clinical relevance insight. Given variations in titers from diverse assay formats, consistent use of the same format and cutoff approach throughout project development is advised. Assays for patient selection are IVDs and must comply with diagnostic guidelines/standards.[61] Regardless of use, a comprehensive assay characterization and understanding of its attributes and limitations are imperative.[61] In cases of varied approaches across development stages, bridging between different methods or laboratories requires careful consideration, utilizing positive control antibodies or AAV Ab-positive samples.

Reference intervals and CDLs used in diagnostic assays

This section outlines the diagnostic assay cutoffs, differentiating between RIs/limits and CDLs and outlining their determination.

RIs help to establish analyte ranges associated with the unaffected, non-diseased state.[62,63] Test results outside an RI do not necessarily indicate disease.[62,63] For instance, although the upper reference limit for fasting blood sugar is 100 mg/dL, a value above 100 mg/dL does not necessarily mean disease. If fasting blood sugar falls between 100 and 125 mg/dL, the subject might belong to a healthy subpopulation with slightly higher levels or an early stage of diabetes mellitus (Figure 1).

In the context of GTs, the RI for preexisting AAV Abs describes the value distribution of a population that is negative for antibodies to the relevant AAV serotype. The upper reference limit is the assay-derived, technical cutoff above which a sample is regarded to contain AAV Abs with no understanding of its functional relevance *in vivo*.

CDLs are established based on prior knowledge of risk for a subject to develop a disease.[62,63] The CDLs are cutoffs for analytes above or below which a subject has a significantly higher risk of an adverse clinical outcome. They can be used to diagnose the presence of a specific disease/condition or to make a treatment decision (Figure 1).

To establish a clinical relevance, the preexisting AAV Ab titer and its association with impact on clinical efficacy or safety will need to be assessed. The relevant CDL would then be a preexisting AAV Ab titer that would have a clinical impact rather than be a technical assay specific cutoff (Table 5).

Definition and determination of RIs

In this section, we outline the definition, a priori determination, and transference and verification of RIs. Additional details can be found in the supplemental information and in the Clinical Laboratory Standards Institute (CLSI) guidelines EP28-A3[57] and EP17-A.[64]

RIs depict the value distribution of an analyte in the unaffected reference population[63] derived from the central 95% range of values in that population (Figure S3).[65,66] To establish the RI with 90% confidence, a minimum of 120 qualified reference individuals for each partition (e.g., sex, age range) are required, as recommended by CLSI EP28-A3.[57] Selection of a reference sample group adequately representing the intended reference population is crucial, and has a strong influence on the outcome of the determined RI.[57] In the GT context, the reference sample group is ideally selected from seronegative individuals of the intention-to-treat (ITT) patient population but availability may be limited and sponsors may use healthy human samples instead. In many cases, this may be a good choice but factors affecting the immune status of a donor such as immunosuppressive treatments, chronic inflammation, and age-related variations in immunoglobulin levels[67–69] can impact the value distribution and thus the RI. Therefore, sponsors must carefully select the reference sample donors and justify their choice (Figure S4) comparing the distribution values of healthy with available ITT donor samples.

While a minority of diagnostic laboratories determine RIs a priori (Figure S4),[57] most of them focus on transference and verification of established RIs/reference limits (Figure S5),[57] and sponsors often determine the upper reference limit during early project phases using their bioanalytical laboratories. When transferring the assay to a diagnostic laboratory in later phases, the diagnostic laboratory verifies the reference limit by analyzing samples and confirming if $\leq$10% of the results are outside the 95% RI of the donor laboratory (Figure S5). The assay is then validated for use as an IVD.[64] In cases where the reference limit is not confirmed, the required cutoff adjustment will affect the patient selection criterion.

In summary, most likely bioanalytical and diagnostic laboratories will be involved in AAV Ab testing, and a close and early collaboration of both fields ensures data consistency throughout development phases and facilitates assay transfer to a diagnostic laboratory, if necessary.

Definition and determination of CDLs

This section covers the clinically relevant CDLs. Additional information is available in the supplemental information and details are described in CLSI EP24-A2.[58]

CDLs usually are defined by consensus groups based on guidelines, consensus values, and clinical outcome studies[62] designed to understand the probability of the presence of a certain disease or a different treatment outcome.[70] In contrary to RIs, which usually are solely based on an unaffected reference population,[65] CDLs consider the distribution of measurement values in both unaffected and affected subjects (Figure 1) and vary among different populations.[66]

CDL determination is a multistep process.[58] Firstly, identify the clinical question: "Which seropositive subjects may have suboptimal therapeutic response to GT?" Define and characterize the ITT population and the clinical decision for patient selection based on AAV Abs. In the second step, select a statistically valid patient sample number for GT treatment independent of their AAV Ab status, including those with incomplete AAV Ab data as this may be an assay characteristic.

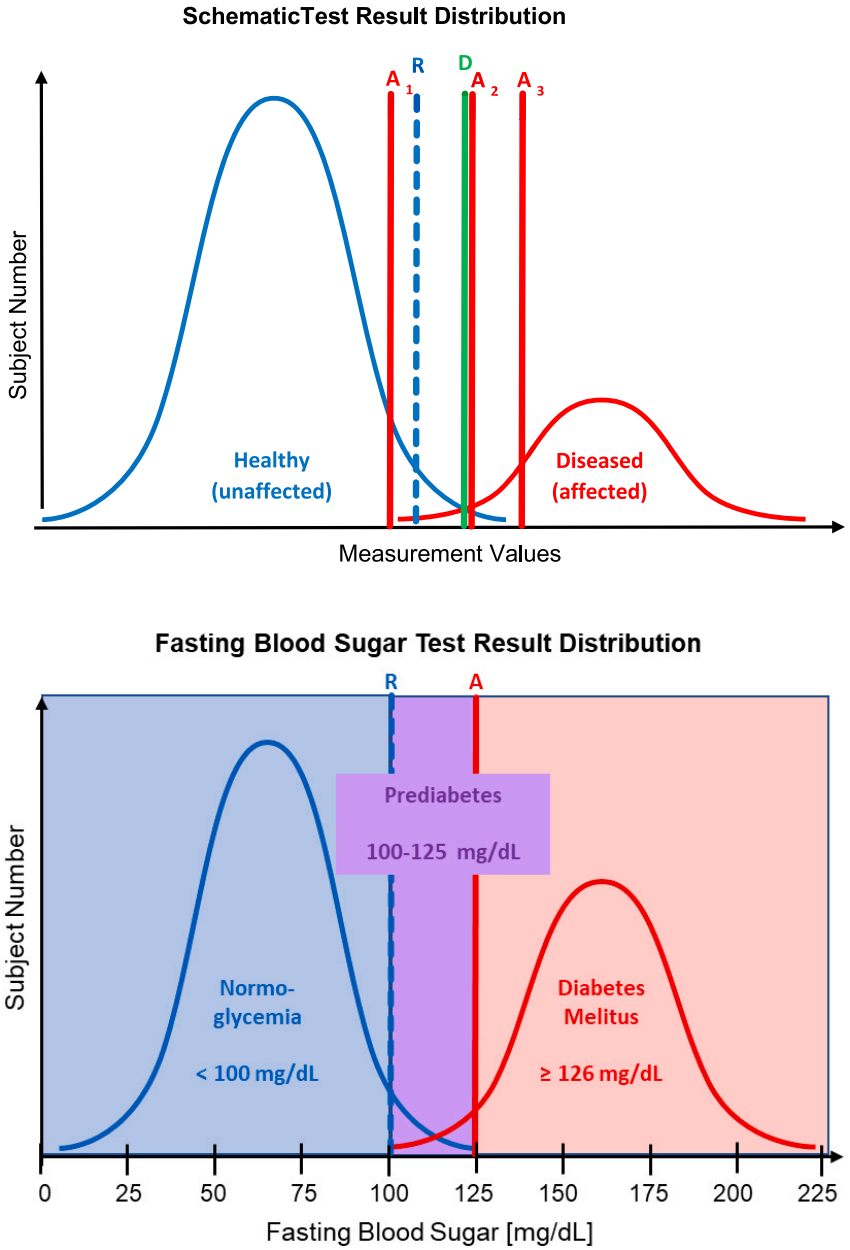

Figure 1. Test result distribution of healthy and diseased subjects

An upper reference limit (R) is determined, for example, using the one-sided 97.5th percentile of the healthy reference population (unimodal consideration). In the diabetes mellitus example, an upper R of 100 mg/dL was determined for the normoglycemic population. In theory, the results of a non-pathological and pathological group do not overlap, and the clinical decision limit (D) clearly separates both groups. However, as shown in the example of diabetes mellitus, 2.5% of normoglycemic subjects and some early-stage patients fall within the range of 100–125 mg/dL fasting blood glucose. Expert groups therefore define action limits (A, A1, A2, A3) and thus determine when a diagnostic or therapeutic measure is necessary. When determining these limits and the associated further diagnostic or therapeutic measures, the risk of misdiagnosis of a healthy and diseased person is weighed against each other (bimodal consideration). For example, A1 can be chosen as the CDL for a rapid SARS-CoV-2 antigen test to limit the spread. A3, on the other hand, is more likely to be chosen for a high-risk procedure such as surgery. In the diabetes mellitus example, only people with a fasting blood sugar level above 125 mg/dL are identified as diabetes mellitus patients and treated therapeutically. However, people in the range of 100–125 mg/dL are monitored diagnostically without therapeutic treatment.

Thirdly, test patients with the AAV Ab assay without knowledge of the patient's clinical classification. Evaluate efficacy/adverse events, using independent external standards for unbiased classification of a patient's "true" clinical state without knowing the patient's AAV Ab status. Compile clinical observations with preexisting AAV Ab test results to determine diagnostic accuracy. The chosen AAV Ab assay must accurately detect AAV Abs for effective patient subgroup classification.

A more detailed description of the CDL determination process can be found in the supplemental information. See Table S3 for an example with a fictive Assay X. Determination of important diagnostic parameters including diagnostic sensitivity (= true positive fraction; proportion of diseased test subjects tested positive), diagnostic specificity (= true negative fraction; proportion of healthy test subjects tested negative), false positive fraction, false negative fraction, predictive positive value (probability that a positive test result is truly positive), and predictive negative value (probability that a negative test result is truly negative) is described in detail in Figure S6 and Figure S7.

To identify CDLs, historical, intervention based, or physiopathological data can be utilized.[71,72] As historical clinical data are not available for GTs and AAV Abs are not linked to the treated disease, CDL determination relies on intervention-based clinical outcome studies. Conducting these studies is challenging in rare diseases with limited patients, common in current GT development.

CDLs for GTs are currently insufficiently determined, relying on a single, predefined, often arbitrary cutoff justified by preclinical studies. Such a CDL, even if it is titer based, may lack clinical relevance, indicating only its use during product development, not guaranteeing impact prevention on efficacy. When seropositive patients with higher titers are included in GT studies using harmonized AAV Ab assays or class LDT/CDx, future CDL definition may involve retrospective clinical data analysis across GTs.

Table 5. Comparison of reference limits with clinical decision limits

	Reference limit	Clinical decision limit
Relevance	statistical limits (upper and lower reference limit); no clinical relevance	clinically relevant cutoff
Purpose	describes the biological characteristic of a reference population	separates subjects into two groups that need different treatment
Reference population(s)	one population:usually healthy, unaffected subjects	two populations:healthy, unaffected subjects and subjects affected with a specific disease
Based on	upper or lower limit of a two-sided 95% central interval of a reference value distribution	clinical outcome studies, guidelines, consensus values
Determined using	parametric (mean + 1.96 × SD or non-parametric approach (97.5th percentile)	ROC curves
Defined by	laboratory experts	laboratory experts in cooperation with clinicians

SD, standard deviation; ROC, receiver operating characteristic.

An ideal CDL balances diagnostic sensitivity and diagnostic specificity, considering clinical consequences of a false-negative or a false-positive AAV Ab test result for a specific GT and patient group (Figures S6 and S7). Currently, clinically relevant CDL determination relies on intervention-based studies treating seropositive and seronegative patients, feasible only by trial sponsors. A diagnostic laboratory developing AAV Ab assays for regulatory approval cannot independently determine a clinically relevant CDL due to limited access to relevant patient populations and clinical data.

How to select an appropriate cutoff for patient selection

In this section, establishment, justification, and potential clinical relevance of cutoffs for patient selection are discussed. One option is to establish a reference limit, excluding patients with AAV Abs above it. Alternatively, a specific titer value above the upper reference limit can be used as the cutoff. Regardless of the chosen approach, the sponsor must determine and justify the cutoff for patient selection, as noted earlier.[57] Refer to Table 6 for pertinent analytical validation parameters and relevant clinical validation studies essential for PMA and humanitarian device exemption (HDE) approval in the USA, with links to corresponding guidance.

Upper reference limit as qualitative cutoff

An upper reference limit, e.g., used in the AAV5 DetectCDx Kit, is justified based on the seronegative reference population and method requirements such as MRD. Test results are usually reported as "seropositive" or "seronegative" without providing a titer and seropositive patients are excluded from GT treatment irrespective of the cutoff's clinical relevance. Using a reference limit is the most conservative patient selection approach minimizing patients' risks but potentially preventing some patients from receiving a beneficial treatment.

Semiquantitative titer as cutoff

If a certain level of preexisting AAV Abs is tolerable, a titer-based cutoff can be justified by preclinical data or prior clinical experience, considering the overall risk-benefit profile. Typically, each sample reports a semiquantitative titer result, and patients are ideally chosen based on a clinically relevant cutoff. This method is potentially riskier, often selected for patients with high unmet medical needs, as seen with the MEBCDX AAV9 test for Zolgensma administration in Japan.

Companies may use published preclinical data to exclude seropositive patients, e.g., above a TI titer of 5. For instance, a study by Wang et al. on AAV8-GFP in non-human primates (NHPs) justified a TI titer of 5 as a patient selection cutoff based on observed effects on vector copy number, GFP expression, and immune response (Table 1).[73]

In a preliminary study using AAV2-FIX intravenously, one patient with a TI titer of 17 had FIX levels reduced to 3%, compared with 11% in a patient with a titer of 2 (Table 1).[74] Lower FIX expression, linked to transaminitis, may result from the destruction of vector-transduced cells by a cellular immune response and reduced transduction of target cells due to preexisting AAV Abs. Employing AAV-GFP or AAV-LacZ reporters, the 50% approach determined TI titers in both cases.[73,74] TAb titers were not provided, but the increase of the vector in the spleen as described by Wang et al.[73] suggests an immune response mediated by various AAV Abs, including TAbs and NAbs.

In a clinical study with AMT-060, higher titers (TI titer $\leq$340, TAb titer $\leq$256) showed no adverse events when AAV5-*wt*hFIX was given to both seropositive and seronegative hemophilia B patients (Table 1).[30] In a corresponding monkey study, TI titers of $\leq$1,030 did not affect transduction or transgene expression after AMT-060 administration, aligning with clinical findings. FIX activity 18 months post-Hemgenix administration was comparable in seropositive and seronegative patients not correlating with preexisting AAV5 TI titers $\leq$678, supporting AMT-060 findings.[30] However, one patient with a TI titer of 3212 showed no human FIX expression (Table 1).[29,31] Considering the limited data, a clinically relevant cutoff for Hemgenix efficacy appears to be between AAV5 TI titers of 1,030 and 3,212, as suggested by NHP data. It is noted that increasing the dose can overcome preexisting AAV Abs impact, as demonstrated in NHPs, potentially affecting translatability to the clinic.[75] The 40-fold lower dose of AMT-060 in NHPs with the highest titer compared with Hemgenix dosing challenges direct translatability.[30,75] In Hemgenix, higher titers were tolerated compared with AAV2 and AAV8 examples, possibly due to serotype differences or reporter sensitivity.

Defining a specific titer cutoff for patient selection requires justification from literature, preclinical, or previous clinical studies, considering assay variations and product parameters. Preclinical studies may better support such cutoffs.[30,76] However, translating preclinical titer/efficacy or titer/safety correlations to the clinic is unclear, requiring more comparative data. Assessing a general correlation between preexisting AAV Abs and safety/efficacy in GT treatment seems feasible with

Table 6. Overview of guidelines/guidance and validation parameters/studies relevant for premarket and humanitarian device exemption approval in the USA

Validation parameters/studies	Brief description	Guideline/guidance
Analytical sensitivity (LoB, LoD, LoQ)	relevant for semiquantitative assays, also recommended for qualitative assays[61]	CLSI EP17-A2 Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures (Protocols For Determination Of Limits Of Detection And Limits Of Quantitation);[64] CLSI EP12-A2 User Protocol for Evaluation of Qualitative Test Performance[95]
Precision	repeatability between, e.g., plates, runs, days, operators, instruments, reagent lots	CLSI EP5-A3 Evaluation of Precision of Quantitative Measurement Procedures;[96] CLSI EP15-A3 User Verification of Precision and Estimation of Bias[97]
"Accuracy"[61] Confirm analytical specificity of the assay for the analyte	demonstrate that a positive test result is related to AAV Abs/activity,[61] e.g., perform a confirmation step to characterize the nature of the positive result	no CLSI guideline[a]
Analytical specificity/interference	endogenous (e.g., hemoglobin, triglycerides, bilirubin)/exogenous (e.g., concomitant medications) potential interferents to include medications common to patient population	CLSI EP07-A2 Interference Test in Clinical Chemistry;[98] CLSI EP07-A3 Interference Testing in Clinical Chemistry;[99] CLSI EP37-Ed1 Supplemental Tables For Interference Testing In Clinical Chemistry[100]
Linearity	relevant for semiquantitative assays; evaluate continuous characteristics of the biomarker across the assay measuring range	CLSI EP06-A2 Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach[101]
High-dose hook effect	relevant for ligand binding assays	CLSI EP34 Establishing and Verifying an Extended Measuring Interval Through Specimen Dilution and Spiking[102]
Cross-reactivity	assess for potentially erroneous results from non-specific products (e.g., antibodies not directed to the AAV); especially relevant for TAb assay	CLSI EP07-A3 Interference Testing in Clinical Chemistry[99]
Cross-contamination/crosstalk between wells	evaluate well-to-well performance within plate; relevant for plate-based assays	
Sample/analyte stability	evaluate sample/analyte stability considering all transport, storage, and handling conditions	
Reagent stability	evaluate shelf-life and in-use stability of the assay reagents	CLSI EP25-A Evaluation of Stability of In Vitro Diagnostic Reagents[103]
Reference limit, seroprevalence assessment	establish/verify reference limit; evaluate a relevant number of individual samples relevant for ITT patient population; assess seroprevalence[61]	CLSI EP28-A3c Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory[57]
Clinical study	clinical validation of CDx	CLSI EP24-A2 Assessment of the Diagnostic Accuracy of Laboratory Tests Using Receiver Operating Characteristic Curves[58]
Bridging study	demonstrate concordance of CDx and IUO/prototype device in case they differ (optional)	Statistical Guidance on Reporting Results from Studies Evaluating Diagnostic Tests[104]

AAV Ab, anti-AAV antibody; IUO, investigational use only; LoB, limit of blank; LoD, limit of detection; LoQ, limit of quantitation; TAb, total antibody.
[a]Performance of an analytical confirmation assay/step is mentioned/described in FDA's immunogenicity guideline and respective white papers.[52,105]

sufficient seropositive and seronegative patients in clinical trials. Yet, identifying a clinically relevant cutoff based on a specific titer value remains challenging due to the need for larger patient populations.

ASSAY JOURNEY FROM PRECLINICAL USE TO CDx

This section outlines the evolution of the AAV Ab assay from preclinical to clinical trials and its ultimate use as a diagnostic assay (Table 7).

Initially, the assay supports preclinical studies, assessing feasibility and establishing a reference limit during method development. It is advisable to use the same assay for both preclinical and clinical phases, ideally adjusting the reference limit for the respective species.

For patient selection, assay transfer to a diagnostic laboratory may be necessary before clinical studies, aligning with national requirements. In the USA, sponsors perform a study risk determination (SRD) to determine if an Investigational Device Exemption (IDE) submission (21 CFR Part 812) is needed before using the assay in clinical studies. A fit-for-purpose validated CTA is typically acceptable for first-in-human trials.

Table 7. Overview of development phases of an AAV Ab assay for patient selection (future CDx) and its use during GT development

	ASSAY USE DURING DEVELOPMENT[a]	ASSAY DEVELOPMENT
PRECLINICAL PHASE	**support preclinical studies**	**preclinical assay development**
	use assay in preclinical studies to support clinical development (e.g., efficacy and toxicology studies)	develop RUO assay
	use same assay format as for clinical studies	• determine reference limit for AAV Ab assessment
	optional: adjust assay to preclinical species (e.g., cut point/reference limit, reagents)	define CDL based on preclinical or previous clinical experience (if required)
	use assay for initial seroprevalence assessments	**CTA development**
CLINICAL PHASE	***observational study/"Natural history study"***	perform SRD to determine if IDE submission is required
	optional trial, in parallel to interventional clinical studies or preclinical phase;	• identify diagnostic laboratory to support analytical assay verification and IDE submission (if required)[a]
	• *characterize the patient population*	transfer assay to diagnostic laboratory able to support patient selection
	• *assess titer range, seroprevalence, seroreversion/seroconversion*	• evaluate assay feasibility
		• finalize assay development according to IVDR in EU and following CLSI standards in USA
	early clinical trials	• validate CTA according to its use (fit-for-purpose)
		start of post launch plannings
	use fit-for-purpose validated CTA (e.g., IUO IVD)	consider patient journey and assay logistics for post-launch phase
		consider approach for reimbursement
	pivotal clinical trials	**finalize CDx development**
	use of the "prototype CDx" (= CTA after design freeze) allows for concomitant approval of the GT and the CDx	complete clinical verification of the CDx assay
	use of the CTA (before design freeze) requires bridging between the CTA and the future CDx potentially delaying GT launch	perform bridging studies between the CTA and the future CDx (if required)
		Crossvalidate the CTA between early and pivotal clinical studies (if required)
POST-LAUNCH PHASE	**Commercialization**	***optional: adjust initial cutoff to clinically relevant CDL***
	use assay according to the drug label	*perform clinical trials including patients with preexisting antibody titers above initial cutoff*

AAV Ab, anti-AAV antibody; CDL, clinical decision limit; CDx, companion diagnostic; CE, Conformité Européenne; CLSI, Clinical & Laboratory Standards Institute; CTA, clinical trial assay; GT, AAV-based GT; IDE, investigational device exemption; IUO, investigational use only; IVD, *in vitro* diagnostic; IVDR, IVD regulation; RUO, research use only; SRD, study risk determination.
[a]Might be required at earlier time point depending on observations in early clinical trials.

Simultaneously, exploratory observational studies may assess seroprevalence and titer value distribution within the relevant patient population, especially if samples from the ITT population are not commercially available.[4,5] These studies may also examine seroconversion rates between sampling and test results availability or patient treatment initiation.[5] In cases involving newborns, understanding the time window for a positive test result to make a patient eligible due to seroreversion can be of interest.[4]

The key phase for regulatory approval of the assay is its use in pivotal phase 3 clinical trials. Ideally, the analytical assay verification of the CTA is completed, and the assay design is locked before the study begins (Table 7). Patients are selected using the CTA after design freeze ("prototype CDx assay"), and the assay is clinically verified. If CTA design freeze is not possible before pivotal studies, a fit-for-purpose validated CTA assay can be used, but this requires bridging studies or cross-validation with the CDx assay, potentially delaying GT launch. An IDE submission may be needed based on previous clinical studies and SRD outcomes. Simultaneously, sponsors address post-launch assay issues, including patient journey, assay and sample logistics, and reimbursement. Post-launch, the assay and GT labels cross-reference each other in various geographies such as the USA, Japan, Australia, and South Korea. Additional trials can adjust the initial cutoff if necessary, however, this requires revalidation and renewed CDx approval.

AAV Ab assay use during preclinical development

In this section, we address AAV Ab assessment during preclinical development, emphasizing considerations for assay transfer to a diagnostic partner before clinical studies, if needed. In the preclinical phase, preexisting AAV Abs are assessed to exclude or randomize animals, determining a relevant cutoff for patient selection. Early studies suggest excluding seropositive animals in product design, but pivotal preclinical studies should ideally mirror clinical situations.

For patient selection, considering the expected human AAV Ab titer range in pivotal preclinical studies is recommended. Administering planned clinical doses to seronegative and seropositive animals allows safety/efficacy comparison, potentially justifying a patient selection cutoff based on observed correlations.

If preclinical species titers do not reflect human titers, administering AAV Abs before GT treatment or inducing them with a low AAV capsid dose can be considered. If patient selection seems unnecessary, randomizing seropositive animals into different treatment cohorts can confirm this assumption, especially for new local ROA assessments.

Ideally, using the same assay and cutoff for preclinical and clinical studies, adjusted for species differences, ensures consistency. Early AAV Ab assay availability aids study design, data interpretation, and cutoff justification, but translatability to the clinic requires further evaluation. This assessment depends on including seropositive patients in clinical studies, which is currently lacking.

AAV Ab assay use during clinical development

This section discusses regulatory requirements for CTAs and the implications of patient selection cutoffs on data interpretation and treatment. Different approaches, such as using the reference limit or a semiquantitative titer cutoff, impact patient eligibility and risk levels. Ideally, a clinically relevant CDL treating all patients is established during clinical development, but challenges arise in including seropositive patients in studies due to potential risks. Adaptive clinical trial designs are recommended to continually modify trial components (e.g., preexisting AAV Abs), allowing determination of a clinically relevant CDL.[77] However, this approach may not be applicable for rare diseases. In some trials, high interindividual variability in transgene protein expression complicates matters,[78–81] and sponsors may prefer avoiding additional factors affecting efficacy.

Regardless of the cutoff, AAV Ab titer information is valuable for physicians and patients. In patient eligibility cases, where titers are slightly above the cutoff, retesting at a later time point can determine eligibility due to intraindividual variability. Seropositive newborns very likely serorevert within a period of a few weeks or months, and the titer values help to define a time interval for retesting.

Any laboratory assay for patient selection or management must comply with IVD and clinical trial regulations, varying globally. In the USA, CAP/CLIA standards apply, with IDE regulation for assays used in clinical trials outside of its intended use or before being approved. SRD must address the impact of false negative results on safety and efficacy as well as ethical issues (e.g., disease severity and SOC) of false positive results. If the risks outweigh the benefit, an IDE application to the FDA is required for significant risk devices (21 CFR 812.3(m)). If the benefits outweigh the risks, patient selection is not necessary. Early engagement with regulatory bodies such as the Center for Biologics Evaluation and Research (CBER) and the Center for Devices and Radiological Health (CDRH) is advised.[82]

EU and UK trials must comply with IVD regulations, using CE marked or in-house developed devices fulfilling IVDR requirements. In the UK, self-certificated UK Conformity Assessed (UKCA) marked devices or validated analytical assays complying with The Medical Devices Regulations 2002 (MDR 2002)[83] can be used. Exemption can be granted by the Medicines and Healthcare products Regulatory Agency on a case-by-case basis. Alternatively, the study must be registered as a performance study for the device. Most other countries require good clinical practice compliance for biomarker testing.[84] In Japan, PMDA guidelines mandate analytical test validation.[85] South Korea requires an approved IDE, and China's regulation prohibits foreign entities from testing clinical study samples, including serum, collected in China.

Diagnostic laboratories must have appropriate accreditations/certifications, such as CAP/CLIA (in USA) and ISO15189 to support patient selection and ISO13485 for PMA submission support.

During clinical development, balancing efficacy demonstration with broad product testing is crucial. A too conservative patient selection cutoff may have ethical implications, excluding patients with high unmet medical needs. Thorough understanding of preexisting AAV Abs' clinical relevance is vital, and early engagement with national authorities is recommended for CTA regulatory requirements.

Registration of the AAV Ab assay in parallel to the GT

In this section, we explore the codevelopment of a CDx with its investigational medicinal product. Ideally, AAV Ab assays for patient selection and the GT gain simultaneous market authorization in jurisdictions with corresponding regulatory requirements. This scenario is supported if the CTA is analytically validated, and the "prototype CDx" is available at the start of the registrational GT trial for clinical validation. Analytical validation ensures the assay's capability to detect the analyte based on predefined performance characteristics. During clinical validation, concordance and efficacy analysis will be used to determine whether the assay is suitable for identifying patients for treatment with the associated GT. Realistically, assays used in clinical trials may change, necessitating bridging between the CTA and the future CDx using archived clinical samples for retrospective analysis. Post-approval, the CDx identifies patients with an acceptable risk-benefit profile for GT treatment in clinical practice.

Various jurisdictions have specific CDx regulations or guidelines, such as EU IVDR,[43] Australian's Therapeutic Goods Administration (TGA) guidelines on IVD CDx,[86] FDA guidance on "In Vitro Companion Diagnostic Devices,"[33] and "Principles for Codevelopment of an In Vitro Companion Diagnostic Device with a Therapeutic Product" (Table S4).[34] In the USA, concomitant approval of the CDx and the therapeutic product is required, but exemptions can be granted for serious or life-threatening diseases lacking satisfactory therapies.

In Japan and South Korea, market application submission must be parallel, as specified in PMDA Notification 1224029, 2013,[85] and Ministry of Health, Labor and Welfare PFSB/ELD Notification

0701-10, 2013.[87] In the EU, China, and Australia, medicinal product and CDx approval are not directly linked. Although not universally required, we recommend striving for concomitant approval of both products globally to prevent inappropriate GT use.

Regulatory requirements for CDx in the USA

To get market authorization as a CDx in the USA, a 510(k), a PMA, or a HDE is required. The 510(k) path is reserved for devices with a legally marketed predicate device. Submitters must compare their device to one or more similar devices and demonstrate substantial equivalence. However, new CDx devices need to follow the more stringently regulated PMA path. In rare indications with fewer than 8,000 people in the USA annually, a device can be placed on the US market without requiring evidence of effectiveness, following the HDE path.

Presubmission meetings can be used to seek agreement on the analytical and clinical studies required for market authorization of the assay (Table 6), on the regulatory submission pathway to achieve FDA market authorization, and on the codevelopment plans for simultaneous crossagency approval of the CDx and GT by CDRH and CBER, respectively.

Regulatory requirements for CDx in the EU, Switzerland, and UK

Since May 2022, the EU has transitioned from the "In Vitro Diagnostic Directive 98/79/EC (IVDD)" to the IVDR. Under IVDD, CDx assays were self-certified with CE marking requiring a technical file aligning with IVDD (now Annex I IVDR). Notified Body review was unnecessary for self-certified assays. The transition to IVDR concluded on May 25, 2022, and now any new assay in the EU market must have CE marking under IVDR. AAV Ab assays for patient selection, falling under the "higher risk—class C," no longer allow self-certification. Instead, a technical file must be submitted to a notified body for review in conformance with IVDR. For CDx conformity assessment, the notified body seeks scientific opinion from EMA before issuing an EU technical documentation assessment certificate, particularly for GTs under the centralized procedure.

The UK, having left the EU, and Switzerland, discontinuing the mutual agreement, follow separate regulatory paths. In the UK, IVDD-based regulations (MDR 2002 No. 618) are still in effect, while the IVD Ordinance (IvDO) in Switzerland aligns with EU principles. A UK regulatory renewal is under discussion, and IVDs placed on the UK market will require a UKCA mark from July 2024.

GTs and CDx are approved by different agencies. In the USA, CBER approves GTs, and CDRH approves CDx. In the EU, EMA centrally approves GTs, while CDx are approved by national authorities, posing a challenge for simultaneous approval of both products.

AAV Ab assay use and requirements for patient eligibility testing after approval

In this section, we address crucial post-launch assay considerations, including market access, reimbursement challenges, competition, sample and data logistics, and clinician education. Early definition of the overall assay strategy, considering regulatory requirements, assay format, and use after launch, is imperative for successful validation and commercialization. Long-term availability, reproducibility, and quality are key requirements, necessitating a robust supply chain of instruments and critical reagents.

Laboratory selection requires device-regulatory expertise and design control validation capabilities. Centralized testing may face challenges in sample shipment, necessitating the ability to transfer assays to other laboratories, if needed.

Evaluation of market access landscapes and timely planning are essential, considering local ordering systems, data transfer security, and compliance with data protection regulations.

Reimbursement alignment for the GT and CDx is ideal but often delayed due to varying decision-makers and legislative disparities.[88] Misalignment may result in GT reimbursement without CDx coverage, prompting sponsors to consider cost-sharing or bundling. However, this may not be viable in all regions, potentially burdening patients or health systems.

A readily available IVD may be used in place of the CDx when multiple IVDs per serotype are available. Given variations in assay formats and analytical sensitivity, this may impact eligibility testing and result in suboptimal treatment of patients. Therefore, clinician education is crucial.

In summary, clinicians, beyond technical requirements, influence assay use and result interpretation, affected by various factors such as market dynamics and logistics.

CONCLUSION AND FUTURE PERSPECTIVES

AAV Abs differ from classical diagnostic biomarkers, serving as a "predictive biomarker."[89] Rather than confirming disease presence, they identify individuals at higher risk of adverse effects from medicine exposure. Various factors, such as product design, immunogenic potential, impurities, therapeutic dose, ROA, immunogenicity mitigation, patient, and disease factors, contribute to the risk of adverse effects beyond preexisting AAV Abs.

A comprehensive risk-benefit assessment is necessary, weighing general patient risks, severity of the underlying disease, available treatments, and medical need against risks associated with administering the GT to a seropositive patient. Excluding all seropositive patients may be preferable for severe diseases with alternative treatments, while less stringent exclusion criteria based on a predefined titer value may be justified for severe diseases with high unmet medical need.

Ideally, during clinical development, a clinically relevant cutoff for patient selection is established by including all patients, regardless of their AAV Ab status, or by incorporating patients with varying titer ranges (e.g., AAV Ab titer <50, 50–250, 250–1,000, >1,000) through an adaptive trial design. Subsequently, the cutoff is determined by

correlating safety/efficacy observations with the corresponding titer ranges. However, conducting such studies is challenging, given uncertainties about the necessary number of dosed seropositive patients and the current limited patient numbers in GT trials. Factors such as high interpatient variability further impede these studies, although improvements may occur with enhanced product designs, manufacturing processes, and immunosuppressive treatments in the future.

The polyclonal nature of antibody responses introduces variability in binding and neutralizing capacity among patients with the same titer, potentially influencing the impact on GT treatment differently. Consequently, establishing a precise titer-based cutoff for patient selection remains challenging, necessitating a risk-benefit assessment to determine the most suitable cutoff within a gray zone. In the absence of clinically relevant cutoff determination in clinical studies, as discussed in this review, the initial patient selection cutoff may need post-launch adjustments, incurring additional costs for CDx development and approval.

The requirement for a CDx according to national regulations depends on the overall risk-benefit profile of the GT and the utilization of the IVD in the study. If the risk-benefit assessment indicates the necessity for patient selection, we recommend early engagement with the relevant authorities, such as CBER and CDRH in the USA. While discussions regarding the need for a CDx should be directed to CBER, pre-submissions can be employed to obtain CDRH feedback on studies supporting future IVD submissions. Unless exempt, all investigational devices are subject to IDE regulations, requiring the performance of an SRD. If the IVD is classified as a significant risk, the sponsor must submit an IDE application, including SRD, to CDRH. Moreover, it is crucial to coordinate PMA/HDE submission with BLA submission as a missing CDx may delay the GT launch. For some patients, such a delay can make treatment impossible because they have developed AAV Abs in the meantime, the disease has already progressed too far, or because the patient has reached an age that is outside the time window within which the health insurance company will cover the treatment costs.

After the launch, the AAV Ab assay for patient selection may not always be specified in the package insert (e.g., Roctavian in EU, Zolgensma in EU and USA, Elevidys), and, in principle, any IVD detecting AAV Abs to the respective serotype can be used, regardless of the assay format. This could lead to the treatment of patients who are unlikely to benefit or have a higher risk for adverse events, given that AAV Ab assays are not comparable between laboratories.

Harmonizing assay formats, validation parameters, procedures, and aligning on best practices for selecting an appropriate MRD and reference samples would enhance the comparability of such assays. Disclosing key assay parameters in IVD labels, field studies, and AAV Ab standards managed and provided by the World Health Organization, coupled with a convention or guideline for reporting titer values, would improve result comparability between various IVDs. This is particularly crucial, as clinicians and patients might opt for a locally available or more affordable IVD than the sponsor's IVD, especially in cases of complicated delivery logistics or unclear reimbursement.

For the reasons discussed in this review, we recommend using the bridging TAb assay as the preferred format for patient selection, establishing a reference limit for titer determination (Table 5),[57] and reporting titer values in reference to the undiluted matrix, encompassing all dilution steps of the sample, as suggested by the FDA for anti-biologic drug antibodies.[52]

An alternative approach to standardize patient selection for a specific AAV serotype could involve the creation of a "class CDx." Unlike classical diagnostic biomarkers, preexisting AAV Abs are not associated with the targeted disease but with the AAV capsid utilized in the GT. It is worth considering whether obtaining companion or complementary diagnostic assay approval for each AAV serotype would be acceptable. A "class CDx" could streamline the number of assays per AAV serotype, contributing to a harmonized and comparable patient selection for GTs utilizing the same serotype as a delivery vehicle.

However, a single patient selection cutoff defined for a "class CDx" might not be universally applicable to all GTs using the respective serotype or all patient populations treated with such GTs. This variation arises from factors such as therapeutic dose, transgene expression efficiency, and ROA impacting the influence of preexisting AAV Abs. In addition, patient conditions, including immune status, may affect the reference limit and thus the patient selection cutoff.

Therefore, we recommend the development and validation of a qualitative "class IVD" incorporating a reference limit to accurately identify seropositivity. Sponsors can then employ this "class IVD" to validate a clinically relevant titer-based cutoff for patient selection in pivotal clinical studies for a particular GT and patient population. Alternatively, a semiquantitative "class CDx," validated and approved for a broad AAV Ab titer range or specific titer-based cutoffs (e.g., 50, 250, 1,000), would enable sponsors to choose the "class CDx" with the most appropriate cutoff for their GT and patient population.

Industrial consortia should engage in discussions with authorities regarding the potential for "class IVDs" or "class CDx" to reduce development time and costs, facilitating earlier patient access to potentially life-saving therapies. This approach ensures patient safety and avoids ineffective treatments.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101217.

ACKNOWLEDGMENTS

The authors would like to thank Kariena Dill (Ultragenyx) for the final review of the article and Anna Laurén (Novo Nordisk) for

discussing the diagnostic guideline requirements. This work received no external funding. The content of this article represents the authors' opinions and does not necessarily represent the views of their employers.

AUTHOR CONTRIBUTIONS

M.B. drafted and finalized the manuscript. All authors have contributed to the contents, review, and references of the article.

DECLARATION OF INTERESTS

The authors are employed by and receive compensation from companies that are involved in development of gene therapy modality therapeutics and are listed on the title page of the manuscript. The authors have no other relevant affiliations or financial involvements with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript. The views and conclusion presented in this paper are those of the authors and do not necessarily reflect the representative affiliation or company's position on the subject.

REFERENCES

1. Mendell, J.R., Connolly, A.M., Lehman, K.J., Griffin, D.A., Khan, S.Z., Dharia, S.D., Quintana-Gallardo, L., and Rodino-Klapac, L.R. (2022). Testing preexisting antibodies prior to AAV gene transfer therapy: rationale, lessons and future considerations. Mol. Ther. Methods Clin. Dev. *25*, 74–83.
2. Wang, D., Tai, P.W.L., and Gao, G. (2019). Adeno-associated virus vector as a platform for gene therapy delivery. Nat. Rev. Drug Discov. *18*, 358–378.
3. Kruzik, A., Fetahagic, D., Hartlieb, B., Dorn, S., Koppensteiner, H., Horling, F.M., Scheiflinger, F., Reipert, B.M., and de la Rosa, M. (2019). Prevalence of Anti-Adeno-Associated Virus Immune Responses in International Cohorts of Healthy Donors. Mol. Ther. Methods Clin. Dev. *14*, 126–133.
4. Calcedo, R., Morizono, H., Wang, L., McCarter, R., He, J., Jones, D., Batshaw, M.L., and Wilson, J.M. (2011). Adeno-associated virus antibody profiles in newborns, children, and adolescents. Clin. Vaccine Immunol. *18*, 1586–1588.
5. Klamroth, R., Hayes, G., Andreeva, T., Gregg, K., Suzuki, T., Mitha, I.H., Hardesty, B., Shima, M., Pollock, T., Slev, P., et al. (2022). Global Seroprevalence of Pre-existing Immunity Against AAV5 and Other AAV Serotypes in People with Hemophilia A. Hum. Gene Ther. *33*, 432–441.
6. Hüser, D., Khalid, D., Lutter, T., Hammer, E.M., Weger, S., Heßler, M., Kalus, U., Tauchmann, Y., Hensel-Wiegel, K., Lassner, D., and Heilbronn, R. (2017). High Prevalence of Infectious Adeno-associated Virus (AAV) in Human Peripheral Blood Mononuclear Cells Indicative of T Lymphocytes as Sites of AAV Persistence. J. Virol. *91*, e02137-16.
7. Wang, D., Zhong, L., Li, M., Li, J., Tran, K., Ren, L., He, R., Xie, J., Moser, R.P., Fraser, C., et al. (2018). Adeno-Associated Virus Neutralizing Antibodies in Large Animals and Their Impact on Brain Intraparenchymal Gene Transfer. Mol. Ther. Methods Clin. Dev. *11*, 65–72.
8. Prasad, S., Dimmock, D.P., Greenberg, B., Walia, J.S., Sadhu, C., Tavakkoli, F., and Lipshutz, G.S. (2022). Immune Responses and Immunosuppressive Strategies for Adeno-Associated Virus-Based Gene Therapy for Treatment of Central Nervous System Disorders: Current Knowledge and Approaches. Hum. Gene Ther. *33*, 1228–1245.
9. Horton, R.H., Saade, D., Markati, T., Harriss, E., Bönnemann, C.G., Muntoni, F., and Servais, L. (2022). A systematic review of adeno-associated virus gene therapies in neurology: the need for consistent safety monitoring of a promising treatment. J. Neurol. Neurosurg. Psychiatry *93*, 1276–1288.
10. Ertl, H.C.J. (2022). Immunogenicity and toxicity of AAV gene therapy. Front. Immunol. *13*, 975803.
11. Chand, D., Mohr, F., McMillan, H., Tukov, F.F., Montgomery, K., Kleyn, A., Sun, R., Tauscher-Wisniewski, S., Kaufmann, P., and Kullak-Ublick, G. (2021). Hepatotoxicity following administration of onasemnogene abeparvovec (AVXS-101) for the treatment of spinal muscular atrophy. J. Hepatol. *74*, 560–566.
12. Chand, D.H., Mitchell, S., Sun, R., LaMarca, N., Reyna, S.P., and Sutter, T. (2022). Safety of Onasemnogene Abeparvovec for Patients With Spinal Muscular Atrophy 8.5 kg or Heavier in a Global Managed Access Program. Pediatr. Neurol. *132*, 27–32.
13. Chand, D.H., Zaidman, C., Arya, K., Millner, R., Farrar, M.A., Mackie, F.E., Goedeker, N.L., Dharnidharka, V.R., Dandamudi, R., and Reyna, S.P. (2021). Thrombotic Microangiopathy Following Onasemnogene Abeparvovec for Spinal Muscular Atrophy: A Case Series. J. Pediatr. *231*, 265–268.
14. Lek, A., Atas, E., Hesterlee, S.E., Byrne, B.J., and Bönnemann, C.G. (2023). Meeting Report: 2022 Muscular Dystrophy Association Summit on 'Safety and Challenges in Gene Transfer Therapy'. J. Neuromuscul. Dis. *10*, 327–336.
15. B Long, T.R., Day, J., Yu, H., Lau, K., Patton, K., de Hart, G., Henshaw, J., Agarwal, S., Vettermann, C., and Gupta, S. (2023). Interim 52-Week analysis of immunogenicity to the vector capsid and transgene-expressed human FVIII in GENEr8-1, a phase 3 clinical study of valoctocogene roxaparvovec, an AAV5-mediated gene therapy for hemophilia A. Hämostaseologie, S34.
16. Jiang, H., Couto, L.B., Patarroyo-White, S., Liu, T., Nagy, D., Vargas, J.A., Zhou, S., Scallan, C.D., Sommer, J., Vijay, S., et al. (2006). Effects of transient immunosuppression on adenoassociated, virus-mediated, liver-directed gene transfer in rhesus macaques and implications for human gene therapy. Blood *108*, 3321–3328.
17. Nonnenmacher, M., and Weber, T. (2012). Intracellular transport of recombinant adeno-associated virus vectors. Gene Ther. *19*, 649–658.
18. Aharoni, S., Bistritzer, J., Levine, H., Sagi, L., Fattal-Valevski, A., Ginzberg, M., Noyman, I., Cohen, R., and Nevo, Y. (2022). Adeno-associated virus serotype 9 antibody titers in patients with SMA pre-screened for treatment with onasemnogene abeparvovec -routine care evidence. Gene Ther. *30*, 101–106.
19. Calcedo, R., Vandenberghe, L.H., Gao, G., Lin, J., and Wilson, J.M. (2009). Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. J. Infect. Dis. *199*, 381–390.
20. Miyazawa, D. (2022). Potential mechanisms by which adeno-associated virus type 2 causes unexplained hepatitis in children. J. Med. Virol. *94*, 5623–5624.
21. Kashiwakura, Y., Baatartsogt, N., Yamazaki, S., Nagao, A., Amano, K., Suzuki, N., Matsushita, T., Sawada, A., Higasa, S., Yamasaki, N., et al. (2022). The seroprevalence of neutralizing antibodies against the adeno-associated virus capsids in Japanese hemophiliacs. Mol. Ther. Methods Clin. Dev. *27*, 404–414.
22. Gorovits, B., Azadeh, M., Buchlis, G., Harrison, T., Havert, M., Jawa, V., Long, B., McNally, J., Milton, M., Nelson, R., et al. (2021). Evaluation of the Humoral Response to Adeno-Associated Virus-Based Gene Therapy Modalities Using Total Antibody Assays. AAPS J. *23*, 108.
23. Gorovits, B., Fiscella, M., Havert, M., Koren, E., Long, B., Milton, M., and Purushothama, S. (2020). Correction to: Recommendations for the Development of Cell-Based Anti-Viral Vector Neutralizing Antibody Assays. AAPS J. *22*, 42.
24. Gorovits, B., Fiscella, M., Havert, M., Koren, E., Long, B., Milton, M., and Purushothama, S. (2020). Recommendations for the Development of Cell-Based Anti-Viral Vector Neutralizing Antibody Assays. AAPS J. *22*, 24.
25. Schulz, M., Levy, D.I., Petropoulos, C.J., Bashirians, G., Winburn, I., Mahn, M., Somanathan, S., Cheng, S.H., and Byrne, B.J. (2023). Binding and neutralizing anti-AAV antibodies: Detection and implications for rAAV-mediated gene therapy. Mol. Ther. *31*, 616–630.
26. Khatri, A., Shelke, R., Guan, S., and Somanathan, S. (2022). Higher Seroprevalence of Anti-Adeno-Associated Viral Vector Neutralizing Antibodies Among Racial Minorities in the United States. Hum. Gene Ther. *33*, 442–450.
27. Luxturna. BLA Clinical Review Memorandum. https://www.fda.gov/files/vaccines%2C%20blood%20%26%20biologics/published/Clinical-Review–December-16–2017—LUXTURNA.pdf.
28. FDA (2020). Guidance for Industry: Human Gene Therapy for Retinal Disorders.

29. Thornburg, C.D. (2021). Etranacogene dezaparvovec for Hemophilia B Gene Therapy. Ther. Adv. Respir. Dis. *2*, 26330040211058896.

30. Majowicz, A., Nijmeijer, B., Lampen, M.H., Spronck, L., de Haan, M., Petry, H., van Deventer, S.J., Meyer, C., Tangelder, M., and Ferreira, V. (2019). Therapeutic hFIX Activity Achieved after Single AAV5-hFIX Treatment in Hemophilia B Patients and NHPs with Pre-existing Anti-AAV5 NABs. Mol. Ther. Methods Clin. Dev. *14*, 27–36.

31. Pipe, S.W., Leebeek, F.W.G., Recht, M., Key, N.S., Castaman, G., Miesbach, W., Lattimore, S., Peerlinck, K., Van der Valk, P., Coppens, M., et al. (2023). Gene Therapy with Etranacogene Dezaparvovec for Hemophilia B. N. Engl. J. Med. *388*, 706–718.

32. Day, J.W., Finkel, R.S., Mercuri, E., Swoboda, K.J., Menier, M., van Olden, R., Tauscher-Wisniewski, S., and Mendell, J.R. (2021). Adeno-associated virus serotype 9 antibodies in patients screened for treatment with onasemnogene abeparvovec. Mol. Ther. Methods Clin. Dev. *21*, 76–82.

33. FDA (2014). In Vitro Companion Diagnostic Devices.

34. FDA (2016). Principles for Codevelopment of an in Vitro Companion Diagnostic Device with a Therapeutic Product.

35. Tosolini, A.P., and Sleigh, J.N. (2020). Intramuscular Delivery of Gene Therapy for Targeting the Nervous System. Front. Mol. Neurosci. *13*, 129.

36. FDA (2020). Guidance for Industry: Human Gene Therapy for Rare Diseases.

37. FDA (2022). Guidance for Industry: Human Gene Therapy for Neurodegenerative Diseases.

38. FDA (2020). Guidance for Industry: Human Gene Therapy for Hemophilia.

39. Treleaven, C.M., Tamsett, T.J., Bu, J., Fidler, J.A., Sardi, S.P., Hurlbut, G.D., Woodworth, L.A., Cheng, S.H., Passini, M.A., Shihabuddin, L.S., and Dodge, J.C. (2012). Gene transfer to the CNS is efficacious in immune-primed mice harboring physiologically relevant titers of anti-AAV antibodies. Mol. Ther. *20*, 1713–1723.

40. Ocana-Santero, G., Diaz-Nido, J., and Herranz-Martin, S. (2021). Future Prospects of Gene Therapy for Friedreich's Ataxia. Int. J. Mol. Sci. *22*.

41. Lee, S., Kang, I.K., Kim, J.H., Jung, B.K., Park, K., Chang, H., Lee, J.Y., and Lee, H. (2019). Relationship Between Neutralizing Antibodies Against Adeno-Associated Virus in the Vitreous and Serum: Effects on Retinal Gene Therapy. Transl. Vis. Sci. Technol. *8*, 14.

42. Day, J.W., Mendell, J.R., Burghes, A.H.M., van Olden, R.W., Adhikary, R.R., and Dilly, K.W. (2023). Adeno-associated Virus Serotype 9 Antibody Seroprevalence for Patients in the United States with Spinal Muscular Atrophy (Molecular Therapy - Methods & Clinical Development), p. 31.

43. EU (2017). Regulation on In Vitro Diagnostic Medical Devices.

44. Scheerens, H., Malong, A., Bassett, K., Boyd, Z., Gupta, V., Harris, J., Mesick, C., Simnett, S., Stevens, H., Gilbert, H., et al. (2017). Current Status of Companion and Complementary Diagnostics: Strategic Considerations for Development and Launch. Clin. Transl. Sci. *10*, 84–92.

45. Jorgensen, J.T. (2021). An update on companion and complementary diagnostic assays for PD-1/PD-L1 checkpoint inhibitors in NSCLC. Expert Rev. Mol. Diagn *21*, 445–454.

46. Twomey, J.D., and Zhang, B. (2021). Cancer Immunotherapy Update: FDA-Approved Checkpoint Inhibitors and Companion Diagnostics. AAPS J. *23*, 39.

47. Dai, Y., Dong, H., Gleason, C., Mora, J., Kolaitis, G., Balasubramanian, N., Surapaneni, S., Kozhich, A., and Jawa, V. (2023). Comparison of Pre-existing Anti-AAV8 Total Antibody Screening and Confirmatory Assays with a Cell-Based Neutralizing Assay in Normal Human Serum. AAPS J. *25*, 35.

48. Falese, L., Sandza, K., Yates, B., Triffault, S., Gangar, S., Long, B., Tsuruda, L., Carter, B., Vettermann, C., Zoog, S.J., and Fong, S. (2017). Strategy to detect pre-existing immunity to AAV gene therapy. Gene Ther. *24*, 768–778.

49. Kasprzyk, T., Triffault, S., Long, B.R., Zoog, S.J., and Vettermann, C. (2022). Confirmatory detection of neutralizing antibodies to AAV gene therapy using a cell-based transduction inhibition assay. Mol. Ther. Methods Clin. Dev. *24*, 222–229.

50. Guideline, E.M.A. (2017). On Immunogenicity Assessment of Therapeutic Proteins.

51. Long, B.R., Sandza, K., Holcomb, J., Crockett, L., Hayes, G.M., Arens, J., Fonck, C., Tsuruda, L.S., Schweighardt, B., O'Neill, C.A., et al. (2019). The Impact of Pre-existing Immunity on the Non-clinical Pharmacodynamics of AAV5-Based Gene Therapy. Mol. Ther. Methods Clin. Dev. *13*, 440–452.

52. FDA. Immunogenicity (2019). Testing of Therapeutic Protein Products-Developing and Validating Assays for Anti-drug Antibody.

53. Shankar, G., Devanarayan, V., Amaravadi, L., Barrett, Y.C., Bowsher, R., Finco-Kent, D., Fiscella, M., Gorovits, B., Kirschner, S., Moxness, M., et al. (2008). Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. J. Pharm. Biomed. Anal. *48*, 1267–1281.

54. Devanarayan, V., Smith, W.C., Brunelle, R.L., Seger, M.E., Krug, K., and Bowsher, R.R. (2017). Recommendations for Systematic Statistical Computation of Immunogenicity Cut Points. AAPS J. *19*, 1487–1498.

55. Shen, M., Dong, X., and Tsong, Y. (2015). Statistical Evaluation of Several Methods for Cut-Point Determination of Immunogenicity Screening Assay. J. Biopharm. Stat. *25*, 269–279.

56. Shen, M., and Dai, T. (2021). Statistical methods of screening cut point determination in immunogenicity studies. Bioanalysis *13*, 551–563.

57. CLSI (2008). Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline—Third Edition. CLSI Document EP28-A3c. Wayne, PA (Clinical and Laboratory Standards Institute).

58. CLSI (2011). Assessment of the Diagnostic Accuracy of Laboratory Tests Using Receiver Operating Characteristic Curves; Approved Guideline—Second Edition. CLSI Document EP24-A2. Wayne, PA (Clinical and Laboratory Standards Institute).

59. Cao, L., Ledeboer, A., Pan, Y., Lu, Y., and Meyer, K. (2022). Clinical enrollment assay to detect preexisting neutralizing antibodies to AAV6 with demonstrated transgene expression in gene therapy trials. Gene Ther. *30*, 150–159.

60. Rogers, G.L., Martino, A.T., Aslanidi, G.V., Jayandharan, G.R., Srivastava, A., and Herzog, R.W. (2011). Innate Immune Responses to AAV Vectors. Front. Microbiol. *2*, 194.

61. Thorne, N. (2023). FDA and ASGCT Workshop on Immune Responses to AAV Vectors: The Regulatory Pathway in the Development of Companion Diagnostic Anti-AAV Assays.

62. Ozarda, Y., Sikaris, K., Streichert, T., and Macri, J.; IFCC Committee on Reference intervals and Decision Limits (C-RIDL) (2018). Distinguishing reference intervals and clinical decision limits - A review by the IFCC Committee on Reference Intervals and Decision Limits. Crit. Rev. Clin. Lab Sci. *55*, 420–431.

63. Ozarda, Y., Higgins, V., and Adeli, K. (2018). Verification of reference intervals in routine clinical laboratories: practical challenges and recommendations. Clin. Chem. Lab. Med. *57*, 30–37.

64. CLSI (2012). Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures; ApprovedGuideline-Second Edition. CLSI Document EP17-A2. Wayne, PA (Clinical and Laboratory Standards Institute).

65. Özcürümez, M.K., Haeckel, R., Gurr, E., Streichert, T., and Sack, U. (2019). Determination and verification of reference interval limits in clinical chemistry. Recommendations for laboratories on behalf of the Working Group Guide Limits of the DGKL with respect to ISO Standard 15189 and the Guideline of the German Medical Association on Quality Assurance in Medical Laboratory Examinations (Rili-BAEK). J. Lab. Med. *43*, 127–133.

66. Sikaris, K.A. (2014). Physiology and its importance for reference intervals. Clin. Biochem. Rev. *35*, 3–14.

67. Khan, S.R., van der Burgh, A.C., Peeters, R.P., van Hagen, P.M., Dalm, V.A.S.H., and Chaker, L. (2021). Determinants of Serum Immunoglobulin Levels: A Systematic Review and Meta-Analysis. Front. Immunol. *12*, 664526.

68. Sitcharungsi, R., Bunupuradah, T., Pornvoranunt, A., Apornpong, T., Ananworanich, J., Khupulsup, K., Nouanthong, P., Vilaiyuk, S., Phasomsap, C., Kamchaisatian, W., et al. (2013). Immunoglobulin values in healthy Thai children aged </= 24 months determined by nephelometry. Asian Pac. J. Allergy Immunol. *31*, 307–313.

69. Bayram, R.O., Özdemir, H., Emsen, A., Türk Dağı, H., and Artaç, H. (2019). Reference ranges for serum immunoglobulin (IgG, IgA, and IgM) and IgG subclass levels in healthy children. Turk. J. Med. Sci. *49*, 497–505.

70. Ali, I.A. (2020). Reference Intervals (RIs), Normal Values, Health Status and Clinical Decision Limits (CDLs), the Theory and the Practice. Journal of Clinical & Community Medicine, 153–154.

71. Sunderman, F.W., Jr. (1975). Current concepts of "normal values," "reference values," and "discrimination values in clinical chemistry. Clin. Chem. *21*, 1873–1877.

72. Abbott, R.D., Wilson, P.W., Kannel, W.B., and Castelli, W.P. (1988). High density lipoprotein cholesterol, total cholesterol screening, and myocardial infarction. The Framingham Study. Arteriosclerosis *8*, 207–211.

73. Wang, L., Calcedo, R., Bell, P., Lin, J., Grant, R.L., Siegel, D.L., and Wilson, J.M. (2011). Impact of pre-existing immunity on gene transfer to nonhuman primate liver with adeno-associated virus 8 vectors. Hum. Gene Ther. *22*, 1389–1401.

74. Manno, C.S., Pierce, G.F., Arruda, V.R., Glader, B., Ragni, M., Rasko, J.J., Ozelo, M.C., Hoots, K., Blatt, P., Konkle, B., et al. (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat. Med. *12*, 342–347.

75. Hurlbut, G.D., Ziegler, R.J., Nietupski, J.B., Foley, J.W., Woodworth, L.A., Meyers, E., Bercury, S.D., Pande, N.N., Souza, D.W., Bree, M.P., et al. (2010). Preexisting immunity and low expression in primates highlight translational challenges for liver-directed AAV8-mediated gene therapy. Mol. Ther. *18*, 1983–1994.

76. Chicoine, L.G., Montgomery, C.L., Bremer, W.G., Shontz, K.M., Griffin, D.A., Heller, K.N., Lewis, S., Malik, V., Grose, W.E., Shilling, C.J., et al. (2014). Plasmapheresis eliminates the negative impact of AAV antibodies on microdystrophin gene expression following vascular delivery. Mol. Ther. *22*, 338–347.

77. Thorlund, K., Haggstrom, J., Park, J.J., and Mills, E.J. (2018). Key design considerations for adaptive clinical trials: a primer for clinicians. BMJ *360*, k698.

78. Konkle, B.A., Walsh, C.E., Escobar, M.A., Josephson, N.C., Young, G., von Drygalski, A., McPhee, S.W.J., Samulski, R.J., Bilic, I., de la Rosa, M., et al. (2021). BAX 335 hemophilia B gene therapy clinical trial results: potential impact of CpG sequences on gene expression. Blood *137*, 763–774.

79. Fong, S., Yates, B., Sihn, C.R., Mattis, A.N., Mitchell, N., Liu, S., Russell, C.B., Kim, B., Lawal, A., Rangarajan, S., et al. (2022). Interindividual variability in transgene mRNA and protein production following adeno-associated virus gene therapy for hemophilia A. Nat. Med. *28*, 789–797.

80. Gray, S.J., Foti, S.B., Schwartz, J.W., Bachaboina, L., Taylor-Blake, B., Coleman, J., Ehlers, M.D., Zylka, M.J., McCown, T.J., and Samulski, R.J. (2011). Optimizing promoters for recombinant adeno-associated virus-mediated gene expression in the peripheral and central nervous system using self-complementary vectors. Hum. Gene Ther. *22*, 1143–1153.

81. Scallan, C.D., Jiang, H., Liu, T., Patarroyo-White, S., Sommer, J.M., Zhou, S., Couto, L.B., and Pierce, G.F. (2006). Human immunoglobulin inhibits liver transduction by AAV vectors at low AAV2 neutralizing titers in SCID mice. Blood *107*, 1810–1817.

82. FDA (2021). Requests for Feedback and Meetings for Medical Device Submissions-the Q-Submission Program.

83. UK (2002). Statutory Instruments: The Medical Devices Regulations 2002.

84. EMA (2016). ICH Guideline for Good Clinical Practice E6(R2)- Step 5.

85. JPMDA (2013). Technical Guidance on Development of in Vitro Companion Diagnostics and Corresponding Therapeutic Products.

86. TGA (2020). Australian Therapeutic Goods Administration: IVD Companion Diagnostics.

87. JPMDA (2013). Notification on Approval Application for in Vitro Companion Diagnostics and Corresponding Therapeutic Products.

88. Govaerts, L., Simoens, S., Van Dyck, W., and Huys, I. (2020). Shedding Light on Reimbursement Policies of Companion Diagnostics in European Countries. Value Health *23*, 606–615.

89. (2016). BEST (Biomarkers, EndpointS, and Other Tools); Silver Spring (MD) (Food and Drug Administration (US)), Resource [Internet]. https://pubmed.ncbi.nlm.nih.gov/27010052/.

90. Wang, L., Bell, P., Lin, J., Calcedo, R., Tarantal, A.F., and Wilson, J.M. (2011). AAV8-mediated hepatic gene transfer in infant rhesus monkeys (Macaca mulatta). Mol. Ther. *19*, 2012–2020.

91. Manno, C.S., Chew, A.J., Hutchison, S., Larson, P.J., Herzog, R.W., Arruda, V.R., Tai, S.J., Ragni, M.V., Thompson, A., Ozelo, M., et al. (2003). AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. Blood *101*, 2963–2972.

92. Mendell, J.R., Sahenk, Z., Lehman, K., Nease, C., Lowes, L.P., Miller, N.F., Iammarino, M.A., Alfano, L.N., Nicholl, A., Al-Zaidy, S., et al. (2020). Assessment of Systemic Delivery of rAAVrh74.MHCK7.micro-dystrophin in Children With Duchenne Muscular Dystrophy: A Nonrandomized Controlled Trial. JAMA Neurol. *77*, 1122–1131.

93. Li, N., and Song, Y. (2022). Strategies for Bottlenecks of rAAV-Mediated Expression in Skeletal and Cardiac Muscle of Duchenne Muscular Dystrophy. Genes *13*, 2021.

94. Donisa Dreghici, R., Redican, S., Lawrence, J., Brown, K., Wang, F., Gonzalez, J., Schneider, J., Morris, C., Shieh, P., and Byrne, B. (2022). IGNITE DMD phase I/II study of SGT-001 microdystrophin gene therapy for DMD: Long-term outcomes and expression update. Neuromuscul. Disord. *32*, S42–S136.

95. CLSI. (2008). User Protocol *for Evaluation of Qualitative Test Performance; Approved Guideline - Second Edition.* CLSI Document EP12-A2 (Wayne, PA: Clinical and Laboratory Standards Institute).

96. CLSI (2014). Evaluationof Precision of Quantitative Measurement Procedures; Approved Guideline - Third Edition. CLSI Document EP05-A3 (Wayne, PA: Clinical and Laboratory Standards Institute).

97. CLSI (2014). User Verification of Precision and Estimation of Bias; Approved Guideline - Third Edition. CLSI Document EP15-A3 (Wayne, PA: Clinical and Laboratory Standards Institute).

98. CLSI (2005). Interference Testing in Clinical Chemistry; Approved Guideline - Second Edition. CLSI Document EP07-A2 (Wayne, PA: Clinical and Laboratory Standards Institute).

99. CLSI (2018). Interference Testing in Clinical Chemistry; Approved Guideline - Third Edition. CLSI Document EP07-Ed3 (R2022) (Wayne, PA: Clinical and Laboratory Standards Institute).

100. CLSI (2018). Supplemental Tables for Interference Testing in Clinical Chemistry; Approved Guideline - First Edition. CLSI Document EP37-Ed1 (Wayne, PA: Clinical and Laboratory Standards Institute).

101. (2004). NCCLS. Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline. NCCLS document EP17-A. In NCCLS, 940 West Valley Road, Suite 1400. Wayne, Pennsylvania 19087-1898 USA.

102. CLSI (2018). Establishing and Verifying an Extended Measuring Interval Through Specimen Dilution and Spiking; Approved Guideline - First Edition. CLSI Document EP34 (Wayne, PA: Clinical and Laboratory Standards Institute).

103. CLSI (2009). *Evaluation of Stability of* in Vitro *Diagnostic Reagents; Approved Guideline.* CLSI Document EP25-A (Wayne, PA: Clinical and Laboratory Standards Institute).

104. FDA (2007). Statistical Guidance on Reporting Results from Studies Evaluating Diagnostic Tests; Availability.

105. Shankar, G., Arkin, S., Cocea, L., Devanarayan, V., Kirshner, S., Kromminga, A., Quarmby, V., Richards, S., Schneider, C.K., Subramanyam, M., et al. (2014). Assessment and reporting of the clinical immunogenicity of therapeutic proteins and peptides-harmonized terminology and tactical recommendations. AAPS J. *16*, 658–673.