BRIEFING DOCUMENT

Food and Drug Administration (FDA)
Cellular, Tissue, and Gene Therapies Advisory Committee
(CTGTAC) Meeting #70

Toxicity Risks of Adeno-associated Virus (AAV) Vectors for Gene Therapy (GT)

September 2-3, 2021

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ABBREVIATIONS

AAV	Adeno-associated virus
AADC	Amino acid decarboxylase
AE	Adverse event
AESI	Adverse events of special interest
ADR	Adverse drug reaction
ADC	Apparent diffusion coefficient
AFP	Alpha-fetoprotein
aHUS	Atypical hemolytic uremic syndrome
ALT	Alanine aminotransferase
ARSA	Arylsulfatase A
AST	Aspartate aminotransferase
BBB	Blood-brain barrier
CBER	Center for Biologics Evaluation and Research
CLN2	Neuronal ceroid lipofuscinosis 2
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
CRISPR	Clustered regularly interspaced short palindromic repeats
CTL	Cytotoxic T lymphocytes
dsDNA	Double-stranded DNA
DNA	Deoxyribonucleic acid
DILI	Drug-induced liver injury
DRG	Dorsal root ganglion
DWI	Diffusion-weighted imaging
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
FIX	Factor IX
FVIII	Factor VIII
GAN	Giant axonal neuropathy
GFP	Green fluorescent protein
GGT	Gamma-glutamyl transferase
GLP	Good laboratory practice
GOI	Gene of interest
GT	Gene therapy
HCC	Hepatocellular carcinoma
HEK293	Human embryonic kidney cells 293
HFD	High-fat diet
HUS	Hemolytic uremic syndrome
IHC	Immunohistochemical
IND	Investigational New Drug Application
INR	International normalized ratio
INTERACT	Initial Targeted Engagement for Regulatory Advice on CBER Products
IT	Intrathecal

ITR	Inverted terminal repeats
IC	Intracranial
ICM	Intra-cisterna magna
ICV	Intracerebroventricular
IV	Intravenous
kg	Kilogram
LTFU	Long-term follow- up
MDA5	Melanoma differentiation-associated protein 5
MPS	Mucopolysaccharidosis type
MRI	Magnetic resonance imaging
MTM1	Myotubularin 1
NCV	Nerve conduction velocity
NHP	Non-human primate
OTAT	Office of Tissues and Advanced Therapies
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline solution
PH	Partial hepatectomy
PI	US prescribing information
PSN	Primary sensory neurons
P/T	Pharmacology/Toxicology
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cells
rcAAV	Replication- competent adeno-associated virus
RD	Regular diet
RNA	Ribonucleic acid
ROA	Route of administration
SAE	Serious adverse event
SC	Spinal cord
SMA	Spinal muscular atrophy
SMN 1	Survival motor neuron 1
ssAAV	Single-stranded AAV
ssDNA	Single-stranded DNA
TALEN	Transcription activator-like effector nucleases
TESAE	Treatment-emergent serious adverse event
TLR	Toll-like receptor
TMA	Thrombotic microangiopathy
TTP	Thrombotic thrombocytopenic purpura
Ub	Ubiquitin
ULN	Upper limit of normal
VEGF	Vascular endothelial growth factor
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
WT	Wild type
XLMTM	X-linked myotubular myopathy
µg	Microgram
vg	Vector genome

1 Introduction

Adeno-associated virus (AAV)-based gene therapies (GT) have shown promise for the treatment of many diseases, including rare diseases with unmet medical needs. In recent years, however, there have been multiple reports of treatment-emergent serious adverse events (TESAEs, serious adverse events that occur after treatment has started) in GT studies with AAV vector-based products (1). These TESAEs include hepatotoxicities, thrombotic microangiopathies (TMA), and brain magnetic resonance imaging (MRI) findings of uncertain significance (2) with some TESAEs resulting in the death of study subjects (3, 4). Other toxicities have largely been reported with AAV vector administration in animal studies. These include dorsal root ganglion (DRG) and peripheral nerve toxicities, which have primarily been characterized in nonhuman primates (NHP) (5, 6).

Oncogenicity due to integration and insertional mutagenesis is also a potential risk of AAV vectors, based on findings of tumors in mice and, more recently, hepatocyte clonal expansion in dogs. Specifically, integration and clonal expansion were noted in the livers of hemophilic dogs many years after administration of an AAV vector, with insertions noted near genes that control cell growth (7). Although AAV vectors have not been shown to cause tumors in humans or nonrodent species, studies in animals indicate a potential for oncogenicity and suggest a need for long-term monitoring.

The emerging knowledge of these risks and toxicities of AAV vectors has led to questions about causality and risk mitigation. The Food and Drug Administration (FDA) is seeking the Committee's insight into strategies to evaluate and mitigate risks in the context of AAV vector-based product design and quality, preclinical studies, and clinical trials.

2 Background

2.1 AAV GT Studies

AAV is a popular vector for gene therapy, accounting for 24% of the viral-vectored GT studies conducted world-wide (8). Over the last decade, the number of clinical studies using AAV vectors has increased rapidly (1). This trend is evident from the submissions received by the Office of Tissues and Advanced Therapies (OTAT) in the Center for Biologics Evaluation and Research (CBER)/FDA (9). From 2015 to 2020, CBER received 99 investigational new drug applications (INDs) for AAV-based GT products, and CBER conducted a large number of pre-IND and Initial Targeted Engagement for Regulatory Advice on CBER Products (INTERACT) meetings to provide advice on future AAV-based GT products (*Figure 1*).

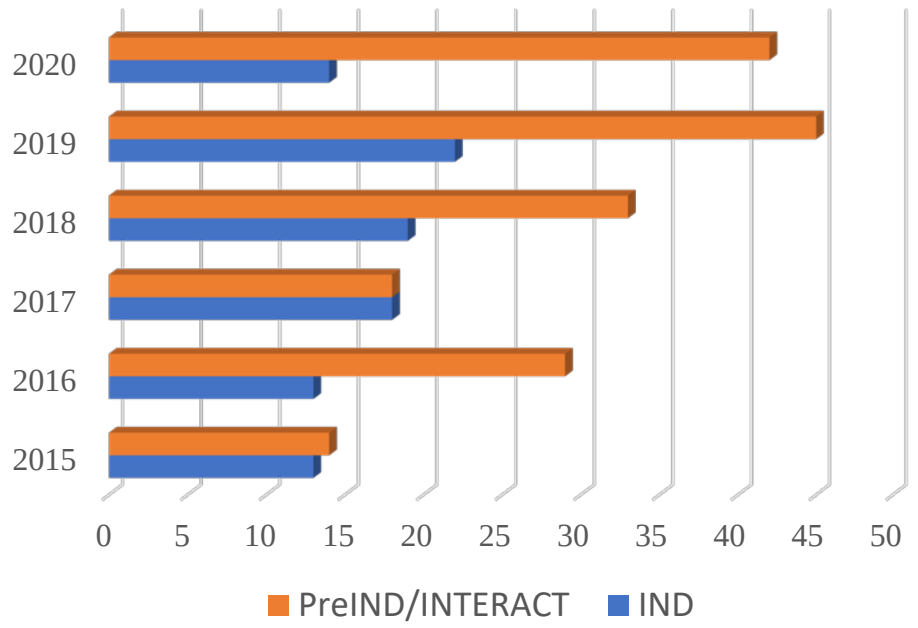


Figure 1 AAV GT product Pre-IND/INTERACT meetings and new INDs in OTAT from 2015 to 2020

Source: Generated by FDA staff from internal database

INTERACT, Initial Targeted Engagement for Regulatory Advice on CBER Products

A number of AAV GT programs have matured to late-phase development, and the FDA has approved two marketing applications for AAV vector-based GT products:

i. voretigene neparvovec-rzyl (Luxturna) – an AAV2 product, administered as subretinal injection, for treatment of patients with confirmed bi-allelic *RPE65* mutations-associated retinal dystrophy (approved in 2017)
ii. onasemnogene abeparvovec-xioi (Zolgensma) – an AAV9 product, administered as a single-dose, intravenous (IV) infusion, for treatment of pediatric patients less than 2 years of age with spinal muscular atrophy (SMA) with bi-allelic mutations in the *survival motor neuron 1 (SMN1)* gene (approved in 2019).

While the number of clinical investigations of AAV vector-based products is increasing rapidly, serious adverse events have also been reported in many studies. In a recent analysis, 35% of 149 AAV GT clinical trials had TESAEs (1).

2.2 AAV Biology and Vectorology

AAV is a popular vector for *in vivo* GT due to the lack of pathogenicity of natural AAV infection and the ability of the AAV genome to persist *in vivo* for a long period of time. AAV vectors are also versatile and can be designed to target particular tissues by:

i. Selecting an appropriate serotype of AAV capsid to preferentially deliver the therapeutic gene of interest (GOI), commonly referred to as the transgene, to the target tissue; and/or
ii. Including tissue/cell-specific regulatory elements (e.g., promoters, enhancers, and/or miRNA targets) that drive expression of the GOI at the target site.

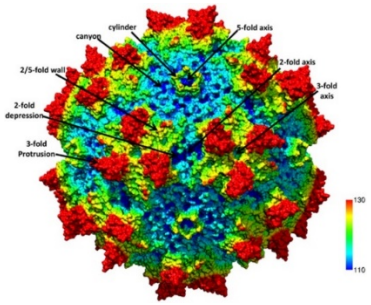
The ability to mix and match different vector elements to tailor a product has greatly increased the utility of these vectors for treating genetic diseases that manifest in specific tissues such as the eye, liver, muscle, peripheral nervous system (PNS) and central nervous system (CNS).

AAV vectors are derived from wild-type (WT) AAVs, which are small (~25 nm diameter) non-enveloped viruses in the genus *Dependovirus* within the *Parvoviridae* family. As the name *Dependovirus* indicates, these viruses depend on a co-infecting helper virus (such as adenoviruses or herpesviruses) to replicate. The WT AAV genome is a single-stranded (ss) DNA of approximately 4.7 kb, with inverted terminal repeat (ITR) sequences that flank two genes, *cap* and *rep* (*Figure 2A*). *Cap* encodes structural proteins that make up the viral capsid. *Rep* encodes replicase proteins that are required for AAV replication and packaging of the genome in the capsid. The ITRs are also essential for replication and packaging and form hairpin-like structures.

In AAV vectors, the *rep* and *cap* genes are removed, leaving only the ITRs flanking the transgene expression cassette(s), which contains the GOI(s) and the regulatory sequences (*Figure 2B*) (10). Manufacturing of AAV GT vectors requires Cap, Rep and helper proteins that are expressed from other DNA sequences. Because AAV vectors lack *rep* and *cap,* they are incapable of replication and packaging *in vivo* (after administration to subjects/patients), even in the presence of helper viruses.

Entry of AAV vectors into cells starts with binding of the AAV capsid to receptors and coreceptors on the cell surface. This interaction is key to the tropism of AAV. Following binding and entry into the cell, the AAV vector genome is uncoated in the nucleus and is maintained extra-chromosomally (episomally), mostly in the form of unintegrated concatemers. In vector-transduced cells, GOI transcription, translation, post-translational modification and translocation are critical for the functionality of the therapeutic protein (*Figure 3)* (11, 12).

A



B

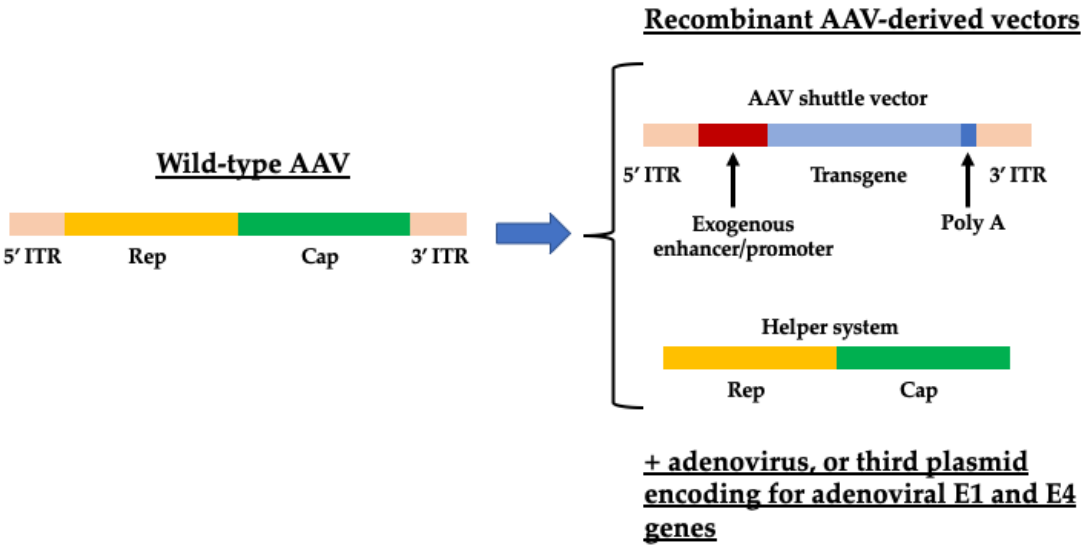


Figure 2

A. The AAV capsid

Source: Tseng and Agbandje-McKenna, 2014. Mapping the AAV capsid host antibody response towards the development of second-generation gene delivery vectors. Front. Immunology 30: 5-9 (13)

B. Schematic representation of wild-type AAV and recombinant AAV-derived vector system

Source: https://www.labome.com/method/Adeno-Associated-Viral-Mediated-Gene-Transfer html (14)

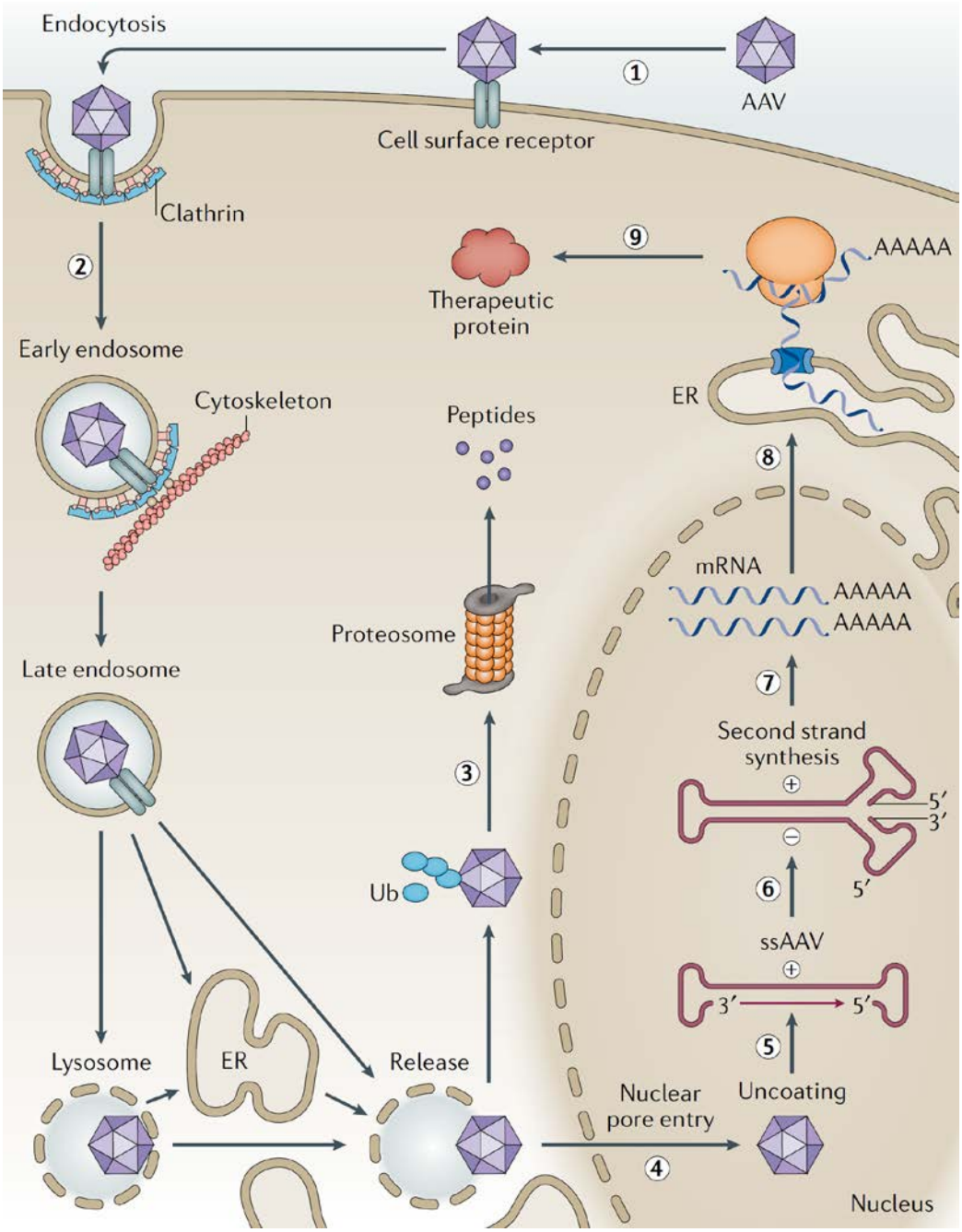


Figure 3 AAV vector transduction pathway

Source: Li and Samulski, 2020. Engineering adeno-associated vectors for gene therapy. Nature Reviews 21: 255-272 (12)

ER , endoplasmic reticulum; ssAAV, single-stranded AAV; Ub, ubiquitin.

Targeting an AAV vector to a particular cell/tissue type is significantly influenced by the choice of the capsid serotype. There are several natural serotypes of AAV, and many of these have been developed as GT vectors, with AAV2, AAV5, AAV6, AAV8 and AAV9 accounting for a majority of the ongoing AAV GT clinical trials (12). Commonly, AAV2 or AAV8 is the choice for ocular therapies; AAV2, AAV8, AAV5, and AAV9 for liver targeting; and AAV2 and AAV9 for muscle and CNS targeting. Some rhesus AAV serotypes such as rh10 and rh74 are also being studied in clinical trials. AAV9 can cross the blood-brain barrier (BBB), allowing IV administration of AAV9 to target brain tissue. Therefore, AAV9 is a popular vector to treat many diseases affecting the nervous system.

The capsids of AAV vectors are derived either from natural AAV viruses or by engineering capsids through rational design, directed evolution or computer-guided strategies. In older children and adults, there is a high prevalence of antibodies against commonly circulating (natural) AAV serotypes. Therefore, the impetus to develop AAV vectors with capsids from less common AAV serotypes or novel engineered AAV capsids is not only to improve or modify tissue targeting, but also to circumvent immune recognition.

2.3 Immune Responses to AAV Vectors and Product Considerations

Although AAV vectors are generally not considered to be proinflammatory, AAV vectors can engage all arms of the immune system: innate, humoral and cellular (*Figure 4*) (15, 16). Immune recognition of AAV vectors can blunt the therapeutic effect of a product and/or raise safety concerns. Immune-mediated toxicities after AAV vector administration have occurred in several clinical trials (15). The role of anti-capsid antibodies, anti-capsid effector T cells, and elicitation of the innate immune mechanisms (including complement activation) are being actively studied, although the determinants of the immunotoxicities in subjects remain mostly undefined.

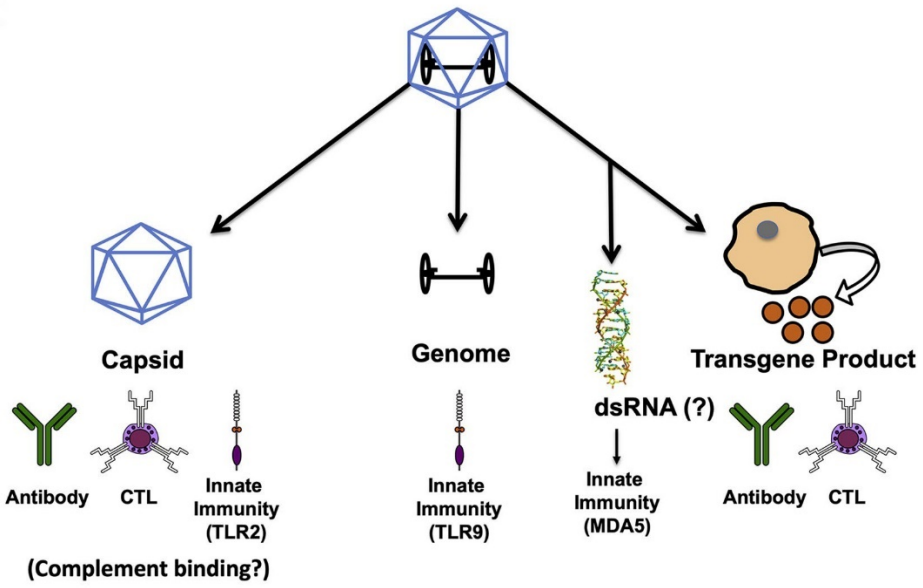


Figure 4 Immune Recognition of the AAV GT vector

Source: Shirley et al., 2020. Immune Responses to Viral Gene Therapy Vectors. Molecular Therapy 28: 709-722 (16)

dsRNA, double-stranded RNA; CTL, cytotoxic T lymphocyte; MDA5, melanoma differentiation-associated protein 5; TLR, Toll-like receptor

As clinical experience with AAV vectors has increased, AAV GT developers have adopted approaches to improve the safety profile and the therapeutic window for these products. These approaches include prophylactic or therapeutic immunosuppression and exclusion of subjects who have high titers of antibodies to the vector. Other approaches include administering high doses of AAV vector to override pre-existing anti-AAV antibodies, and/or avoiding systemic exposure by administering the product locally (e.g., muscle) and/or to tissues/organs such as the CNS and eye that are generally regarded as immune-privileged.

Some clinical trials have administered high doses of AAV GT product, ~ 10^{13} to 10^{14} vector genomes (vg) per kilogram (kg) of body weight (*Table 1*) (17). Inflammatory toxicities, including renal and hepatic toxicities, attributed to such high doses have been reported in clinical studies (*Table 2*) (18). A correlation between dose and vector immunogenicity has also been noted (19, 20). However, it is difficult to compare vector genome doses across clinical studies due to a lack of standardized reference materials and differences in the design and accuracy of assays (21).

Table 1: Highest doses of AAV vector administered in different GT clinical trials

NCT Number	GT Product	Vector Serotype	Indication	Max Dose (vg/kg)	Company
NCT03769116	SRP-9001	AAV-rh74	DMD	2×10^{14}	Sarepta
NCT04652259	AMT-061	AAV5	Hemophilia B	2×10^{13}	uniQure
NCT03392974	BMN-270	AAV5	Hemophilia A	4×10^{13}	BioMarin
NCT03199469	AT-132	AAV8	XLMTM	3×10^{14}	Audentes
NCT04406277	Zolgensma	AAV9	SMA	1.1×10^{14}	Novartis
NCT04468742	SGT-001	AAV9	DMD	2×10^{14}	Solid Biosciences
NCT03362502	PF-06939926	AAV9	DMD	3×10^{14}	Pfizer

Source: Modified from Nicole Paulk; July 7, 2020 in commentary: Gene Therapy: It's Time to Talk About High Doses; https://www.genengnews.com/commentary/gene-therapy-its-time-to-talk-about-high-dose-aav/ (17)
SMA, spinal muscular atrophy; XLMTM, X-linked myotubular myopathy; DMD, Duchenne muscular dystrophy.

Table 2: Some of the severe adverse events reported in AAV GT trials

Condition	Vector Serotype	Dose (vg/kg)	Severe Adverse Event
SMA1	AAV9	6.7×10^{13} to 1.1×10^{14}	Elevated liver enzymes, acute liver injury, thrombocytopenia, TMA
DMD	AAV9	5×10^{13} to 3×10^{14}	Thrombocytopenia, anemia, complement activation, acute kidney injury, cardiopulmonary insufficiency, persistent vomiting
XLMTM	AAV8	1×10^{14} to 3×10^{14}	Gastrointestinal infection, elevated troponin, hyperbilirubinemia, liver failure, sepsis, death

Source: Modified from Nicole Paulk; July 7, 2020 in commentary: Gene Therapy: It's Time to Talk About High Doses; https://www.genengnews.com/commentary/gene-therapy-its-time-to-talk-about-high-dose-aav/ (17)
SMA, spinal muscular atrophy; XLMTM, X-linked myotubular myopathy; DMD, Duchenne muscular dystrophy.

AAV vectors contain significant levels of impurities such as empty capsids (vector particles devoid of the genome) and residual plasmid DNA (used in manufacturing). However, the effects of such impurities on the safety of AAV vectors are not well understood (17, 22, 23). Some mechanistic studies point to the contribution of empty capsids to the elicitation of capsid-reactive lymphocytes, e.g., in subjects with liver enzyme elevation post-treatment in hemophilia trials (24), as further described under Section 2.4 of this document. In addition to capsid impurities, the vector genome itself carries immune triggers: specifically, CpG dinucleotides (25, 26), and the double-stranded form of the vector genome that can be sensed by innate immune mechanisms

upon AAV entry into the cell (27). Residual plasmid DNA that can be packaged in AAV capsids may also be a trigger for innate immunity (28).

Overexpression of the GOI in the target tissue may lead to deleterious effects on normal cellular functions or processes. This was noted recently in SMNΔ7 SMA mice, where an AAV vector that overexpresses SMN protein caused neurodegeneration (29). This finding highlighted the importance of vector design and the choice of regulatory elements that are key to fine tuning gene expression *in vivo*.

Thus, vector design, vector manufacturing and product-specific attributes are key considerations when investigating the determinants of toxicities, and the complex interplay between these factors remains poorly defined.

2.4 Immune Mechanisms and the Role of Vector Attributes (Quality)

The role of product (AAV vector) and process (manufacturing)-related impurities as potential triggers of immune-mediated toxicities has been discussed in recent studies, raising concerns about the quality of the AAV vectors used in clinical studies, as described below.

2.4.1 Empty Capsids

AAV empty capsids, composed of an AAV capsid shell but lacking the vector genome (nucleic acid molecule packaged within), are found in variable proportions in clinical-grade AAV vector preparations (30). The empty capsids increase overall antigenic load and potentially exacerbate capsid-triggered innate and adaptive immune responses. The empty capsids can contribute to the peptides presented by major histocompatibility complex (MHC) molecules, with consequent recognition and clearance of transduced cells by capsid-specific cytotoxic T cells (28). AAV vector-mediated liver toxicity observed in subjects in hemophilia clinical studies, as indicated by transaminase elevations, was associated with activation of capsid-specific CD8+ T cells and with subsequent decline in Factor VIII (FVIII) and Factor IX (FIX) activity (31-33). The AAV capsid is also thought to function as a pathogen-associated molecular pattern (PAMP) that can be

recognized by toll-like receptor (TLR) 2, resulting in the induction of innate immune responses (34, 35). In addition to stimulation of innate and adaptive immune responses, AAV empty capsids may compete with full capsids for receptor binding on target cells, which could necessitate an increase in the required vector dose (36).

In a few studies, purified empty capsids were intentionally mixed with the AAV vector and studied as decoys for anti-AAV antibodies to enhance gene transfer and, thereby, to circumvent the problem of pre-existing humoral immunity (37). In most cases, however, the presence of empty capsids in clinical formulations is undesirable. Elimination of empty capsids can potentially improve the safety margin when high vector doses are administered in clinical studies. Removal of AAV empty capsids during manufacture of the AAV vector is challenging when scale-up is needed for commercial manufacturing (38). However, reduction of empty capsids to very low levels is achievable, and advances in AAV vector manufacturing designed to optimize downstream purification methods have shown improvements in vector quality with reduced proportion of empty capsids (28).

2.4.2 CpG Motifs

CpG motifs in DNA consist of cytosine followed by guanine, and the methylation status of the cytosine affects activation of innate immunity. Hypomethylated CpG motifs in AAV genomes (39) activate the TLR9 innate pathway and trigger cytotoxic T lymphocyte (CTL) formation in mice (40-42). In hemophilia B GT studies, stronger CTL responses and absence of durable transgene expression were noted with AAV vectors that had a higher CpG content (28). Strategies proposed to circumvent CpG-mediated immune activation include codon modification to remove CpGs, increasing CpG methylation by improved production technologies, and antagonizing TLR9 activation by co-expression of short non-coding DNA sequences to "cloak" the much larger AAV DNA sequence (28, 43).

2.4.3 Encapsidated Non-Vector DNA

AAV vector preparations carry many process- and product-related impurities, including non-vector DNA impurities that are packaged in capsids. AAV capsids can package heterogenous fragments of DNA derived from the production cells and from helper components such as plasmids or viruses (also described in Section 3.2.3 of this document) (38, 44). The packaged nucleic acid is protected by the AAV capsid against nuclease digestion (used during manufacturing) and is impossible to remove or reduce to a smaller size. Depending on the production system used, the DNA contaminants in AAV preparations can be fragments of (i) genomic DNA derived from packaging cells, (ii) the plasmid backbone, (iii) helper viruses such as adenovirus, herpesvirus or baculovirus, (iv) WT AAV *rep*/*cap* sequences, or (v) truncated AAV genome (30). The immune-related risks associated with encapsidated non-vector DNA impurities include the potential to express immunogenic peptides and the presence of additional TLR9 agonist CpG motifs derived from helper plasmids (30).

2.4.4 Other Vector Attributes Implicated as Immune Triggers

Additional product attributes may influence AAV-mediated toxicities. AAV vectors carrying self-complementary DNA (scDNA; presented as double-stranded DNA) may be more proinflammatory than vectors carrying single-stranded DNA (ssDNA); higher levels of TLR9-mediated innate immune responses were noted in livers of mice treated with AAV vectors carrying scDNA than in mice treated with AAV vectors carrying ssDNA (27).

2.4.5 Vector Attributes: Controls in Manufacturing and Lot Release

AAV vectors with different design elements (e.g., different vector genome conformations (scDNA or ssDNA), regulatory elements (e.g., constitutive or tissue-specific promoters), and capsid type (different serotypes)) are currently in clinical development for a wide range of indications. Manufacturing approaches are also varied, e.g., AAV vectors are manufactured in insect cells or mammalian cells, as adherent or suspension cells, and empty capsids may or may not be removed during purification. Such differences in vector design and manufacturing

approaches may lead to differences in product attributes that may play a role in triggering the toxicities associated with AAV vectors.

AAV vectors contain several process- and product-related impurities, as noted earlier in this section. The safe limits for most of these impurities are unknown. However, to ensure the safety and efficacy of AAV vectors, FDA recommends that sponsors control for all known impurities as part of in-process testing during manufacturing and as part of testing during product lot release (45). For early-phase development, sponsors should, at a minimum, measure the levels of impurities in clinical vector lots and demonstrate similar purity between lots used in IND-enabling preclinical studies and clinical lots. Per 21 CFR 211.165(a) and 610.13, for licensure and for release of post-licensure lots, manufacturers are required to establish and justify appropriate limits for impurities in their product, based on clinical and manufacturing experience.

2.5 Characterizing Risks in Pharmacology/Toxicology Studies

In accordance with all development programs for investigational products, it is important that data generated from the conduct of Pharmacology/Toxicology (P/T) studies for AAV vector products inform the product benefit-risk profile within the context of the target clinical indication (46). A well-designed preclinical testing program for an AAV vector product includes comprehensive studies that are designed to identify and characterize any product-mediated toxicities based on the intrinsic product attributes, putative mechanisms of action of the product, biological relevancy of the animal species/model, and the proposed clinical indication/target patient population.

As highlighted in Section 2.3 of this document, the toxicity of AAV vectors in clinical studies is dose dependent. Published preclinical data emphasize the importance of administering the anticipated clinical dose levels in animal toxicology studies to better understand AAV vector-mediated dose-response relationships (47-49). Likewise, the route of administration (ROA) and *in vivo* vector biodistribution and tropism play important roles in understanding the potential for on- and off-target tissue-specific toxicities (40, 50-54).Thus it is important that

preclinical toxicology study designs incorporate these factors to mimic the clinical scenario as closely as possible.

Based on the biological properties of AAV vectors and available data highlighting a multitude of factors that may influence associated risks (5, 6, 29, 55, 56), the selection of an animal species/model that is biologically relevant to the target patient population is important. The tropism of different AAV vector capsids (57-61) and host immune responses against the vector (62, 63) can affect the level and duration of exposure to the AAV vector product and, thereby, play an important role in the selection of an appropriate animal species. The occurrence of potential immune-related toxicities can also vary among animal species (64). Genetic differences across species, age and/or pre-existing tissue pathology (55) are also thought to impact risks such as AAV-mediated oncogenicity. In addition, testing in an animal model of disease/injury that mimics relevant aspects of the pathophysiology of the medical condition of the target clinical population may allow for a better understanding of the dose/toxicity relationships of an investigational AAV GT. Finally, the ROA and product delivery procedure can also influence species selection (65-68).

For each P/T study, the parameters evaluated and the timing of these assessments, both in-life and post-mortem, are intended to characterize the type, causative nature, onset, progression and potential resolution of local and systemic findings following product administration (46). These study design elements are important considerations to comprehensively characterize the safety profile of AAV vector products.

In view of the AAV GT vector-associated toxicities (e.g., hepatotoxicity, DRG toxicity, TMA, vector-mediated oncogenicity) reported in animals and/or clinical studies in recent years, understanding the most appropriate types of P/T studies and study design elements is critical to evaluating the risks associated with AAV vectors.

2.6 Clinical Safety Evaluations for AAV Vectors

Clinical safety evaluation can be divided into short-term monitoring (e.g., the first 1-2 years following AAV vector administration) and long-term monitoring (more than 1-2 years following AAV vector administration).

During the short-term monitoring, because immune responses to AAV vector may pose important safety risks, immunoassays measuring cellular and humoral immune responses to both the vector and the transgene-encoded protein have been used to monitor for systemic immune reactions. Other monitoring includes periodic clinical, laboratory and imaging assessments.

For long-term monitoring, the FDA Guidance, "Long Term Follow-Up After Administration of Human Gene Therapy Products" (January 2020) considers the long-term persistence of AAV vectors and the clinical experience with these vectors, and recommends a risk-based approach for determining the duration of the long-term follow-up (LTFU) protocol (69). For example, an LTFU period of up to 15 years is recommended when AAV vectors carry genome editing components. The recommended duration of follow-up for all other AAV GT products is up to 5 years, due to the lower risk of AAV vector integration, in comparison to integrating viral vectors such as lentiviral or other retroviral vectors. The objective of such observational studies is to identify potentially delayed adverse events, e.g., the risk of malignancy, impairment of gene function, and autoimmune-like reactions (69).

For the two marketed AAV vector products, voretigene neparvovec (Luxturna) and onasemnogene abeparvovec (Zolgensma), the planned observational period for study subjects who completed the Phase 1 or Phase 3 clinical trials is 15 years. Post-marketing data from these approved products, as well as additional safety data and LTFU data generated from ongoing clinical trials (and also from related long-term preclinical studies) for a variety of AAV vectors, should inform FDA and sponsors on the type and duration of LTFU observations to ensure the development of safe and effective AAV-vectored GTs.

3 AAV Vector Integration and Oncogenicity Risk

3.1 Introduction

Recombinant AAV-based GT vectors can integrate into the genomic DNA of both animals and humans. Vector-mediated insertional mutagenesis (also referred to as genotoxicity) and hepatocellular carcinoma (HCC) were first reported in neonatal mucopolysaccharidosis (MPS) type VII mice (70) and were caused by integration events in the *Rian* locus that encodes numerous regulatory RNAs (71). Subsequent studies with AAV vectors demonstrated similar integration events and HCC development in different neonatal mouse models (47, 55, 72). More recently, clonal expansion of hepatocytes was reported in a canine model of hemophilia A following AAV vector administration (7), although tumor formation was not observed. Taken together, these animal studies suggest the potential for oncogenicity due to AAV integration, but thus far AAV integration has not been associated with the occurrence of cancer in nonrodent species or humans. Notably, there are also no reports of oncogenesis associated with AAV integration in non-hepatic tissues. A multitude of factors can influence the incidence of AAV vector-mediated oncogenicity and impact risk assessment of AAV vectors, as described in published studies and highlighted in the following sections.

3.1.1 Integration of Wild Type AAV

WT AAV can integrate into genomic DNA and establish latency. AAV integration is mediated by the Rep proteins, which can bind, unwind and nick DNA (73). It is important to note that AAV GT vectors lack Rep and integrate into DNA less efficiently than WT AAV, as explained further in Section 3.1.2 of this document.

Integration of AAV is driven by the ability of Rep to bind specific DNA sequence elements (tandem GAGC/T sequences) in both the viral and genomic DNA. In the genome of the most extensively studied AAV serotype, AAV2, Rep-binding elements are present in the p5 promoter and the ITRs (74-76). Of note, AAV2 contains a liver-specific enhancer–promoter element near the 3’ ITR (77), but there is currently no information on whether this element might enhance the

risk of AAV-induced oncogenicity in the liver. Both the p5 promoter and the liver-specific enhancer–promoter element are deleted in AAV GT vectors, but the ITRs remain because they are essential *cis* elements for replication and packaging of vector DNA.

AAV integration sites can be difficult to fully map because integrated AAV genomes are often concatemeric, partially deleted or rearranged. AAV2 preferentially integrates at a locus in human chromosome 19, but the percentage of integration events at this site varies significantly among cell types, and AAV2 integration has been identified at many thousands of other sites (78-80). Comprehensive mapping studies in cultured human cells show that WT AAV2 tends to integrate in open chromatin regions near consensus Rep-binding elements (78, 79). Many of these integrations occur near transcriptionally active genes, and one study identified integration events near 29 known proto-oncogenes (79).

A study published in 2015 identified integrated fragments of AAV2 DNA in HCC tumors from patients who had not received GT (81). This study had methodological limitations and was insufficient to establish WT AAV as a causal factor for HCC (82-84). More recent work from multiple independent groups has confirmed clonal AAV insertions in a small percentage of HCC cases – AAV insertions were located near proto-oncogenes in some of these tumors, and the insertions were associated with elevated transcription of these proto-oncogenes (85-88).

Although these studies of HCC establish that a small percentage of HCC have WT AAV integrations, the studies are inconclusive regarding a causal role for AAV in HCC. Moreover, as described further below, there are considerable differences between AAV vectors and WT AAV. Therefore, the integration patterns and oncogenic potential of WT AAV have limited relevance to evaluating the risks of GT with AAV vectors.

3.1.2 Integration of AAV Vectors

Following transduction of cells and tissues with *rep*/*cap*-deleted AAV vectors, the AAV vector genome persists mostly in the form of circular non-integrated DNA concatemers (89), but AAV vectors can also integrate into genomic DNA (90). Integration of AAV vectors has been detected

in both animals and humans (7, 91-97). Similar to WT AAV, integration of AAV vectors often involves concatemerization, partial deletion or rearrangement of the vector genome (7, 71, 91, 97). Interestingly, in human recipients of AAV GT, AAV vector integration has been found not only in nuclear DNA, but also in mitochondrial DNA (93).

Because AAV vectors lack *rep*, the integration sites for AAV vectors are distributed more randomly throughout the genome than WT AAV integration sites, and integration of AAV vectors is less efficient than WT AAV integration. Most studies find that AAV vectors preferentially integrate into regions of open chromatin near genes that are being actively transcribed, and AAV vectors do not show any preference for integrating near Rep-binding elements (78, 93, 94, 96, 98). It has been proposed that AAV vectors can integrate by nonhomologous end-joining at spontaneous chromosomal breaks (99). In other words, even though AAV vector integrations are frequently mapped to chromosomal breaks, deletions or translocations (98, 100), it is possible that AAV vectors may not always be the original cause of the chromosomal damage. However, there is also evidence that integrated ITRs are unstable and can increase chromosomal damage (101).

3.2 Risk of Insertional Mutagenesis: AAV Vector Design and Quality Attributes

There are at least four aspects of AAV vector design or quality that might influence the risk of oncogenicity. 1) Vector serotype influences tropism and biodistribution; 2) Enhancer–promoter elements in an integrated vector can transactivate nearby cellular proto-oncogenes; 3) DNA impurities (e.g., *rep*, replication-competent AAV, or viral oncogenes) packaged in vector capsids could increase oncogenesis; 4) The use of AAV vectors that carry genome editing components can increase vector integration rates.

3.2.1 Vector Serotype

As highlighted in Section 2 of this document, the serotype of the AAV vector can determine tropism and *in vivo* biodistribution, thereby influencing tissue-specific disposition of the vector and subsequent transduction efficiency. As a result, the vector serotype may have an important

influence on the risk of AAV vector-mediated oncogenicity. For example, a recent study conducted in neonatal Twitcher mice demonstrated HCC development following administration of AAV9 vector into the CNS, while a similar study using an AAV5 vector did not find any HCC development (12, 102). The authors hypothesize that, in contrast to the AAV5 vector, the AAV9 vector may cross the blood-brain barrier and leak into systemic circulation after CNS administration, with subsequent trafficking to the liver, resulting in HCC.

3.2.2 Enhancer-Promoter Strength

Studies in mice show that HCC can be caused by AAV vector-mediated transactivation of proto-oncogenes (47, 71, 72, 103-105). Even though these tumors in mice are mostly caused by vector integration in a locus (*Rian*) that is not present in the human genome, these results nevertheless demonstrate that AAV vectors can induce insertional oncogenesis. A study conducted in a neonatal mouse model of methylmalonic acidemia demonstrated that vectors with strong enhancer–promoters, such as the ubiquitous chicken β-actin promoter, were more likely to transactivate proto-oncogenes and cause tumors (47).

A similar relationship between enhancer–promoter strength and insertional oncogenesis in animals is well established for retroviral GT vectors, including self-inactivating lentiviral vectors (106, 107). Studies of retroviral vectors have also defined other mechanisms for insertional oncogenesis that might plausibly apply to AAV vectors, including loss-of-function by insertional inactivation of tumor suppressor genes, as well as complex gain-of-function effects on nearby proto-oncogenes via aberrant splicing or read-through of transcripts from the vector (106-111). Unlike retroviral vectors, AAV vectors integrate less efficiently; yet there is the potential that these different vectors cause insertional oncogenesis by similar mechanisms. Studies of lentiviral vectors in tumor-prone mice suggest that careful vector design can decrease – but not eliminate – the risk that vector integration will lead to oncogenicity (106).

3.2.3 Encapsidated DNA Impurities

During assembly of AAV vectors, Rep binds to the 3' ITR of the vector genome and packages the vector genome into preformed capsids. However, non-vector sequences (including plasmid DNA, helper virus DNA, and DNA from the host cells used during vector manufacturing) can also be packaged (112-114). AAV vector genomes can recombine with non-vector DNA via both homologous and non-homologous recombination, and bacterial sequences from vector plasmids can also be packaged by a mechanism known as "reverse packaging." Thus, AAV vector preparations contain many chimeric sequences that consist of a vector ITR attached to non-vector sequences (114). One study demonstrated long-term persistence of these DNA impurities after administration of AAV vectors to animals (115), although the study did not examine whether the impurities were integrated into genomic DNA.

The amount of encapsidated non-vector DNA in AAV preparations is highly dependent on the vector design and manufacturing method. For example, when vector is manufactured by transient transfection of plasmids in human cells, up to 26% of the resulting capsids may contain plasmid DNA (116), although 1-3% is more typical (112, 114, 115). After exposure of cells or mice to AAV vectors, chimeric AAV/plasmid sequences have been found integrated in genomic DNA (100, 117). In addition to capsids that contain plasmid DNA, a few percent of capsids contain human DNA fragments that are acquired randomly from the host cells used to manufacture the vector (112, 114). While it is often possible to quantitate the amounts of various DNA impurities in AAV vector preparations, it is currently not known whether these impurities significantly increase the risk of oncogenicity. Therefore, it is difficult to set risk-based limits for these impurities.

Considering how DNA impurities might theoretically affect oncogenicity, one sequence of concern is the *rep* gene, which is used in all AAV vector production systems. Rep is a concern because of its helicase and endonuclease activities, which might increase the mutation rate and enhance vector integration in transduced cells (118). In addition, if *rep*/*cap* from a packaging plasmid gains an ITR by recombination during manufacturing, a replication-competent AAV (rcAAV) may be formed that will be able to express Rep and replicate in the presence of a helper

virus (119). Two such helper viruses, Human Herpesvirus 6 and Epstein-Barr virus, are commonly found in the human liver (87). During release testing, AAV products are routinely assessed for the presence of rcAAV, but rcAAV assays have limited sensitivity.

Viral oncogenes are the other major type of DNA impurity that might theoretically increase risk of tumor formation. Importantly, viral oncogene sequences are typically short enough to fit the packaging constraints of AAV capsids. Cells that contain viral oncogenes are commonly used to manufacture AAV vectors. Examples include HEK 293 cells (adenovirus E1), HEK 293T cells (adenovirus E1 and SV40 large T antigen) and HeLa cells (human papillomavirus E6 and E7). Adenovirus is used as a helper virus in some manufacturing methods, and plasmids that contain the adenovirus E4 region are used in the popular “triple transfection” method of AAV vector production. The adenovirus E4 region encodes proteins that can contribute to transformation by several mechanisms, including inhibiting the tumor suppressor p53 (120). In cultured cells, the adenovirus E1 and E4 genes can transform cells in a “hit-and-run” mechanism, and persistence of these adenoviral genes is not essential for maintenance of the transformed state (121). This means that it may not always be possible to identify whether a viral oncogene was the original transforming event for a tumor. Therefore, overall, there is insufficient information to evaluate possible oncogenicity risks from the use of viral oncogenes during the manufacturing of AAV vectors.

3.2.4 Genome Editing Components

AAV vectors are increasingly being used for genome editing, carrying genome editing components and/or as DNA donors for homologous recombination. Even without using any genome-targeted nuclease such as clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) or transcription activator-like effector nucleases (TALENs), AAV vectors are often more efficient donor templates for homologous recombination than other forms of DNA (122, 123). When used together, genome-targeted nucleases and AAV vector donor templates synergize to produce relatively high levels of genome editing (124). However, because AAV vectors can integrate at chromosomal breaks,

genome-targeted nucleases that produce double-strand breaks can also lead to high levels of unwanted AAV vector integration (125-127).

3.3 Risk of AAV Vector-Mediated Oncogenicity in Animal Studies

3.3.1 Impact of Animal Species and Disease Pathology

Comprehensive assessments of AAV-mediated oncogenesis have not been reported in rodent species other than mice, and tumor development has, thus far, not been reported in nonrodent species following AAV vector administration. As a result, the predominance of available animal data demonstrates that AAV-mediated oncogenesis can occur in mice via integration into the *Rian* locus (128). While this locus is only found in rodents, similar genomic regions exist in humans and nonrodent species; however, the impact of integration events at these sites is unclear. Because AAV vectors are thought to integrate randomly into transcriptionally active regions of the genome, it is not surprising that integration events have also been observed outside of the *Rian* locus and near proto-oncogenes (7, 12, 104, 129). In a recent study in juvenile dogs with hemophilia A, hepatic clonal expansion was reported approximately 7-10 years following IV administration of AAV8 or AAV9 vectors expressing canine FVIII (7). While no signs of nodule formation or oncogenesis were observed in any of these dogs, in the analyzed clones, integration events were found at genes associated with transformation in humans. No integration events were identified in the *Rian–Dlk–Dio* region (chromosome 8).

Differences in animal age have also been observed to influence AAV vector-mediated oncogenesis. For example, the incidence of HCC development following AAV vector administration in juvenile and adult mice was reported to be lower than in neonatal mice (94, 104, 105, 130, 131). One contributing factor to this difference may be that the *Rian* locus is more transcriptionally active during early stages of murine development (128). Likewise, it has been suggested that the rapid growth rate of the liver in neonatal mice may facilitate AAV vector integration and subsequent HCC development, compared to healthy juvenile and adult animals that have a lower rate of hepatocyte turnover (55).

Moreover, published data indicate that disease pathology can also have an impact on this risk. The influence of pre-existing pathology on AAV vector-mediated HCC was explored in a recent study in which liver damage was induced in adult mice using either a high-fat diet (HFD) or partial hepatectomy (PH) (55). Following systemic administration of two different AAV vectors, an increased incidence of HCC (50-100%) was noted in mice on an HFD compared to animals on a regular diet (RD), and all mice that underwent PH developed tumors. Adult male animals subjected to an HFD or PH also had increased hepatocyte growth similar to or above levels observed in neonatal mice. Subsequently, given the decreased hepatic growth rate in female animals on an HFD compared to male animals, the authors also showed that estrogen administration in male mice partially ameliorated liver inflammation and slowed the hepatocyte growth rate. The authors hypothesized that increased hepatocyte proliferation, coupled with inflammation, contributed to a higher incidence of HCC. This suggests that the underlying liver pathology may impact the development of AAV vector-mediated HCC in mice via integration events in the *Rian* locus. In addition, there may be a potential for sex-specific differences in AAV-mediated oncogenesis within the context of pre-existing liver pathology.

Overall, while tumor formation has only been reported in mice, the available data demonstrate the potential for AAV vectors to integrate into highly active genes, some of which are associated with cell growth, proliferation and/or transformation. However, the identification of relevant animal species/models to characterize the risk of AAV-mediated oncogenesis for investigational AAV-based GT products remains unclear.

3.3.2 Assessments and Duration of Follow-up after Administration of AAV Vector Products

The identification and characterization of tumors in animals following AAV vector administration relies on the use of sensitive, accurate and reproducible in-life and post-mortem assessments over a sufficiently long study duration. Various approaches to evaluate AAV vector-mediated oncogenesis have been reported, including: 1) histopathological assessments to characterize the type of lesion, 2) potential markers associated with HCC (e.g., elevated serum alpha-fetoprotein (AFP)), and 3) gene expression profile and vector integration analysis of healthy and tumor tissue (7, 12, 47). Different methodologies for evaluating vector integration

patterns have been reported. However, given the significant truncation and rearrangement, episomal presence, and low frequency of AAV vector integration, it has been challenging to conduct comprehensive, high resolution integration analysis (7, 91, 93). Additionally, while genetic differences may be a contributing factor to the varying incidence of AAV vector-mediated oncogenesis among species, the duration of follow-up after AAV vector product administration is also important. Due to the long-term persistence of AAV vectors and reports of hepatocyte clonal expansion (7) or HCC development later in life in dogs and mice respectively, life-long monitoring of animals following dosing may be informative. In nonrodent species, follow-up durations of 8-10 years in various canine disease models (7, 132, 133), 8 years in MPS type VI cats (103), and 4 weeks to 6 years in healthy NHPs (91, 97, 134-136) have been reported. Given the significant lifespans of these species, it is unclear whether these animals were evaluated long enough to adequately determine the incidence of AAV vector-induced tumors. It is important to highlight that the lifespan of an animal will vary with the species, strain, and disease/injury condition, as well as the corresponding influence of AAV vector administration on survival.

3.4 Evaluating the Risk of AAV-Mediated Oncogenicity in Clinical Studies

In comparison to other viral vectors with the potential for insertional mutagenesis (i.e., retroviral vectors derived from gammaretrovirus, lentivirus, and foamy virus), AAV vectors have been generally considered safer alternatives for gene delivery. Despite the concerns for genotoxicity of AAV vectors, stemming from animal studies (as described in Section 3.3 of this document), to date there has been only one reported case of HCC in a participant of a clinical trial (HOPE-B Phase 3 Trial for hemophilia B, NCT03489291) who received AAV5 vector for treatment of hemophilia B. Over 50 participants in the HOPE-B trial have been exposed to this GT product, and all had abdominal ultrasound one year after receiving the treatment (137). Further investigations and follow-up analysis of the biopsied samples from the subject concluded that it is unlikely that the AAV vector contributed to the subject's HCC, i.e., there was no clear association between the occurrence of HCC and insertional mutagenesis by the AAV vector (138).

3.5 **Advisory Committee DRAFT Discussion Questions**

1. Please discuss the merits and limitations of animal studies to characterize the risk of AAV-mediated oncogenicity, and provide recommendations on specific preclinical study design elements, to include:
    a. Animal species; healthy vs. disease models; and animal age
    b. In-life and terminal assessments, including methods for integration analysis
    c. Duration of follow-up post-administration

2. Current literature suggests that various factors may affect AAV vector genome persistence, vector integration and the risk of oncogenesis. Please discuss benefit-risk considerations for AAV vector-mediated oncogenesis, such as patient's age at the time of treatment, pre-existing liver conditions (e.g., hepatitis B and C viral infection), and high vector dose.

3. Considering the risk of oncogenesis,
    a. Please provide recommendations on safety monitoring measures that should be included in clinical studies.
    b. Please provide recommendations on duration, frequency, and method of long-term follow-up (LTFU) for recipients of AAV vectors.

4. Please discuss whether some vector designs may enhance the frequency of vector-mediated integration and the risk of oncogenesis. For example, how is the risk affected by promoter-enhancer elements, genome-targeted nucleases, or novel AAV vector designs for which there is limited clinical experience. Because AAV vectors can carry significant levels of co-packaged DNA impurities from the manufacturing process, is the risk of oncogenesis increased due to potential integration of non-vector DNA, and what types of studies should IND sponsors perform to assess this risk?

4 Hepatotoxicity

4.1 Hepatotoxicities Observed in Humans

Nearly all AAV vectors traffic to the liver when delivered IV, and hepatotoxicity is the most common adverse event in clinical trials where AAV vectors are administered IV. This hepatotoxicity often presents as liver enzyme elevation, i.e., elevations of serum alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST), and less frequently as drug-induced liver injury (DILI). Rare cases of hepatic failure and three fatalities have been reported in AAV vector clinical trials (3, 4). Corticosteroids have been used to dampen AAV-mediated hepatotoxicities. The following sections provide a summary of hepatotoxicity data in humans with SMA, X-linked myotubular myopathy (XLMTM) and hemophilia.

4.1.1 Spinal Muscular Atrophy (SMA)

SMA is an autosomal recessive neurodegenerative disorder resulting from irreversible loss of lower motor neurons in the brainstem and spinal cord secondary to bi-allelic mutations of the *SMN1* gene, which results in a paucity of SMN protein. SMA is the most common monogenic cause of infant mortality, with an incidence of 4-10 per 100,000 live births. SMA is classified into 5 subtypes based on age at onset and greatest motor function achieved. The most common phenotype, SMA type 1, is observed in approximately 45–60% of cases. Phenotype is influenced by the nearby gene *SMN2:* additional copies of *SMN2* correlate with reduced severity of disease. Pediatric patients less than 2 years of age with SMA most commonly have two copies of *SMN2*, developing SMA type 1. Such infants may appear normal at birth, but within 6 months typically develop severe flaccid paralysis; they do not achieve developmental milestones such as the ability to sit independently, and mortality is about 30% at age 2 years, with approximately half of those survivors fully reliant on noninvasive ventilation (139).

To date, over 800 patients with SMA have received the recommended IV dose (1.1×10^{14} vg/kg) of onasemnogene abeparvovec (Zolgensma) before or after its approval; all 800 had low titers of anti-AAV9 antibodies (< 1:50) prior to the treatment (140). Among those 800 patients,

approximately one third had at least one adverse event of hepatotoxicity. Typically, hepatotoxicity associated with onasemnogene abeparvovec presents as non-cholestatic (i.e., elevations in serum aminotransferases), most often occurring between one week and one month after product administration. Some patients had serum aminotransferase elevations as high as over 20 times the upper limit of normal (ULN), as described below. In most cases, liver enzyme concentrations normalized following treatment with corticosteroids. Some patients needed treatment with corticosteroids extending as long as 229 days (141).

Prior to approval of onasemnogene abeparvovec, one patient with SMA type 1 who received onasemnogene abeparvovec in an expanded access program, developed acute serious liver injury. At baseline, the patient had elevated AST and ALT of unknown etiology; other liver function indicators (gamma-glutamyl transferase, total bilirubin, and prothrombin time) were normal. Approximately 7 weeks after onasemnogene abeparvovec infusion, the patient developed jaundice. Laboratory testing was consistent with acute liver failure: AST approximately 80 × ULN; ALT approximately 45 × ULN; total serum bilirubin approximately 4 × ULN; and plasma prothrombin time approximately 4 × ULN. Liver biopsy showed acute massive degeneration of hepatocytes, and massive mixed inflammatory infiltrate (primarily CD8-positive T lymphocytes). Patient's liver function recovered to baseline with prednisolone treatment (142).

After marketing approval of onasemnogene abeparvovec in 2017, there have been additional case reports of severe hepatotoxicity. Two patients with SMA type 1 who received onasemnogene abeparvovec met the diagnostic criteria for DILI using Hy's law, whereby the drug causes (1) hepatocellular injury (ALT or AST ≥3-fold above the ULN), (2) elevated serum total bilirubin >2 x ULN, and (3) there is no known alternative cause of liver damage (such as viral hepatitis, ischemia or another drug capable of causing the observed injury) (143). Brief description of the two case reports is presented below:

One patient was a 6-month-old infant with SMA type 1 (0 copies of *SMN1*, 2 copies of *SMN2*) and presented 7 weeks after treatment with onasemnogene abeparvovec (1.1×10^{14} vg/kg; 6.25×10^{14} vg), with irritability, jaundice, international normalized ratio (INR) of 5.3, increase in

aminotransferases (~50-100 × ULN) and direct hyperbilirubinemia. His baseline serum aminotransferase levels were elevated (~5-10 × ULN), and baseline bilirubin was normal. An anti-AAV9 antibody titer before treatment was <1:25 (negative). Evaluation for infection revealed norovirus in the stool. Liver biopsy showed massive ballooning degeneration with drop-out of hepatocytes in zone 3, extensive inflammation in the periportal areas (composed mainly of CD8+ T cells, with some neutrophils, eosinophils, and plasma cells), a marked bile ductular reaction with neutrophilic periductular inflammation, and moderate periportal and marked central vein fibrosis.

The other patient was a 20-month-old child with SMA type 1 (0 copies of *SMN1*, 2 copies of *SMN2*), and presented 8 weeks after treatment with onasemnogene abeparvovec (1.1×10^{14} vg/kg; 11.5×10^{14} vg), with irritability, jaundice, INR of 1.5, rise in aminotransferases (~ 40-50 × ULN) and direct hyperbilirubinemia. Evaluation for infection revealed norovirus in the stool. Before receiving onasemnogene abeparvovec, her ALT, AST, gamma-glutamyl transferase (GGT), and total bilirubin levels were within normal limits and the anti-AAV9 antibody titer was negative. She had received prednisolone 2 mg/kg/day 1 day before treatment, on the day of infusion, and for 2 days after the infusion, followed by 1 mg/kg/day until Day 14 post-infusion when her prednisolone was increased to 2 mg/kg/day due to elevated aminotransferases (~50-100 × ULN). Despite the increased dose of prednisolone, her liver enzymes continued to rise. Liver biopsy revealed expanded portal triads and mild interface hepatitis composed mainly of neutrophils and CD8+ T cells, with occasional CD4+ T cells, CD20+ B cells, and eosinophils. Bile ducts had reactive changes with proliferation, confirmed by CK7 staining, with no acute cholangitis. The hepatic lobule had a lymphocytic infiltrate composed primarily of CD8+ T cells. Masson trichrome stain demonstrated mild portal fibrosis with occasional thin fibrous septae extending into the lobule with mild lobular pericellular fibrosis. There was diffuse hepatocellular swelling involving the entire lobule, with rare ballooning.

Both patients were hospitalized and treated with methylprednisolone 20 mg/kg/day for 3 days followed by gradual taper. Both patients were discharged home on oral prednisolone following clinical and laboratory improvement in liver function (143).

Because of the potential serious risks associated with hepatotoxicity, a Boxed Warning is included in the US Prescribing Information (PI) for onasemnogene abeparvovec to alert prescribers to the possibility of acute serious liver injury and elevated aminotransferases. In addition, the PI includes instructions on administration of systemic corticosteroids equivalent to oral prednisolone at 1 mg/kg of body weight per day, starting one day prior to onasemnogene abeparvovec infusion for a total of 30 days and monitoring of liver enzymes and function by clinical examination and laboratory testing.

4.1.2 Hemophilia

The main toxicity seen in hemophilia clinical studies of AAV-based GT has been early transient elevation in liver enzymes that is mostly asymptomatic (144). Elevation of aminotransferases in these subjects was associated with AAV capsid-specific cell-mediated immune responses. Some subjects developed elevation in aminotransferases without detectable anti-capsid immune responses, while others had an anti-capsid immune response without an elevation in aminotransferase levels (145).

Loss of transgene expression (i.e., vector-mediated expression of clotting factors) was noted in some subjects with the onset of liver enzyme elevations. The presence of anti-capsid T cell responses in a subset of these subjects suggests that the observed loss in clotting factor expression may have been due to T cell-mediated loss of transduced hepatocytes (31, 32, 136, 146-148). Elevation of liver enzymes (in subjects treated with AAV vectors for hemophilia), however, is not always associated with the loss of transgene expression or with the detection of anti-capsid cellular immunity, as noted in the hemophilia B study with AAV5 expressing WT FIX (149, 150).

4.1.3 X-Linked Myotubular Myopathy (XLMTM)

XLMTM is a serious, life-threatening, and rare neuromuscular disease that is characterized by extreme muscle weakness, respiratory failure, and early death. Mortality rates are estimated to be 50 percent in the first 18 months of life, and for those patients who survive past infancy, there is

an estimated 25% mortality by the age of 10 years. XLMTM is caused by *myotubularin 1* (*MTM1*) gene mutations that lead to a lack or dysfunction of myotubularin, a protein that is needed for normal development, maturation, and function of skeletal muscle cells. The disease affects approximately 1 in 40,000 to 50,0000 newborn males.

More than 80% of XLMTM patients require ventilator support, and the majority of patients require a gastrostomy tube for nutritional support. In most patients, normal developmental motor milestones are delayed or never achieved. Currently, only supportive treatment options, such as ventilator use or a feeding tube, are available.

As of July 8, 2020, 23 subjects had received IV administration of an investigational AAV-based GT product, AT132 (AAV8 vector expressing *MTM1* gene) in the ASPIRO trial (NCT03199469). Six subjects received low dose AT132 at 1×10^{14} vg/kg of body weight, and 17 subjects received a high dose of 3×10^{14} vg/kg. The median age of subjects in the low-dose and high-dose cohorts was 1.7 years (range 0.8-4.1) and 2.6 years (range 0.6-6.8), respectively. Three subjects in the high-dose cohort died approximately 20-40 weeks after receiving AT-132. The treatment protocol included prednisolone 1 mg/kg/day for the first eight weeks post-treatment; prednisolone was slowly tapered over Weeks 9-16. By the time of death, all three subjects were older than 5 years of age. An investigation to determine whether these fatal adverse events were related to systemic administration of AT132 was initiated. It was noted that these subjects were among the older and heavier patients in the ASPIRO trial and received the highest total dose (range 4.8-7.7 $\times 10^{15}$ vg). Subjects in the low-dose cohort and younger and lighter subjects in the high-dose cohort did not have similar, treatment-related serious cholestatic liver dysfunction (151). Also, all three subjects who died had pre-existing hyperbilirubinemia as evidence of intrahepatic cholestasis. Autopsy findings in these three subjects included intrahepatic and canalicular cholestasis, periportal and bile ductular reaction, secondary fibrosis, and lack of prominent liver parenchymal inflammatory cellular infiltrates. In these three fatal cases, the subjects presented with an unusual clinical picture of hepatotoxicity, notable for increases of predominantly direct bilirubin up to 35-50 × ULN (with top direct bilirubin levels of 30-90 × ULN). In contrast, AST/ALT elevations typically lagged weeks to months behind the bilirubin elevation. Treatment with ursodiol, augmented corticosteroids and various

immunosuppressants were ineffective in halting the injury progression in these cases. The immediate cause of death was sepsis in two cases and gastrointestinal bleeding in the other case (17, 152).

4.2 The Role of Disease-Related Factors in Hepatotoxicity

In an observational natural history study, 33 patients with XLMTM, mean age 10 years and 11 months, were followed for one year. Hepatic involvement was reported as abnormal liver enzymes or function test (22.5%), enlarged liver (11.8%), jaundice (14.7%) and liver hemorrhage (5.9%) (153). In the ASPIRO trial, more than 50% of participants had some evidence of pre-existing hepatobiliary disease including intermittent direct hyperbilirubinemia, intermittent transaminase elevation, and/or historical cholestasis or jaundice yet no similar, treatment-related, serious cholestatic liver dysfunction was observed in the six subjects in the 1×10^{14} vg/kg cohort or in any of the younger, lighter subjects in the 3×10^{14} vg/kg cohort, regardless of pre-existing hepatobiliary disease.

While there is no clear causal link between hepatotoxicity and the three fatalities in the ASPIRO trial, there was dose-related hepatotoxicity, especially in the presence of pre-existing elevated liver enzymes in children with XLMTM. Pre-existing hyperbilirubinemia could be due to increased red blood cell (RBC) osmotic fragility and hemolysis. Also, some patients with XLMTM may develop peliosis hepatitis, a liver condition characterized by multiple randomly located, blood-filled cavities throughout the liver (17). The preliminary autopsy report for one subject showed multiple well-circumscribed, brown-red lesions throughout the liver, consistent with hepatic peliosis. However, the exact mechanism of deaths in the participants of this clinical trial remains unknown (154, 155).

4.3 Findings in Animal Studies

Liver-associated adverse findings have been reported in different animal species/models following AAV vector administration. Select published reports detailing hepatotoxicities and associated findings of thrombocytopenia and coagulopathy in animals are summarized below.

These data suggest that the findings may be influenced by a number of factors, including the animal species/model, dose levels, vector construct, and other study design elements. It is noteworthy that many of the animal safety studies described here did not assess the impact of immunosuppression on liver toxicity, while clinical studies in subjects with SMA and hemophilia included the administration of prophylactic corticosteroids in an attempt to limit loss of transgene expression (147, 156) and elevation of liver enzymes (157, 158).

4.3.1 SMA

Adverse findings in the liver have been reported in animals administered AAV vectors intended to treat neuromuscular disorders, including SMA (159). Neonatal FVB/NJ mice administered onasemnogene abeparvovec IV at dose levels greater than 1.1×10^{14} vg/kg displayed transient elevated liver enzymes (ALT, AST) levels in the course of a 12-week interval post-dose. Microscopic observations of minimal to moderate hepatocyte degeneration/necrosis, and minimal to slight hepatocellular hypertrophy, perinuclear vacuolation, and increased Kupffer cells were also reported (141).

IV administration of an AAV9 variant serotype (AAVhu68) vector encoding for the *SMN* gene (AAVhu68-hSMN) in three NHPs at a dose level of 2×10^{14} vg/kg resulted in marked elevation in serum liver enzymes (ALT, AST, GGT, alkaline phosphatase). One of the three NHPs became hypothermic and had pale mucous membranes, hepatomegaly, dyspnea, dilated pupils, and signs of circulatory shock; the animal was subsequently euthanized within 5 days after vector administration. The liver tissues were observed to harbor significantly higher (1000-fold) vector genome concentrations compared to other organs and tissues. Additionally, histopathology showed diffuse liver enlargement, massive hepatocellular necrosis and degeneration, immune infiltration, fibrin deposition, and thrombi formation. The remaining two NHPs sacrificed at 4 weeks post-dose exhibited transient elevated serum ALT levels, thrombocytopenia and liver regeneration, fibroplasia, and mononuclear cell infiltration in addition to minimal multifocal hepatocellular necrosis in the periportal areas of the liver (5, 160).

Based on their onset, the authors attributed these hepatic findings to the AAV vector and not an adaptive immune response to the vector. DNA damage/endoplasmic reticulum (ER) stress responses which can be activated by transduction with ultra-high dose levels of AAV vectors and constitutive transgene overexpression in hepatocytes were also proposed as possible mechanisms for the observed toxicity. Additionally, marked elevation of inflammatory cytokines at Day 4 post-dose and the induction of innate immune responses (to the AAV vector) may have elicited or exacerbated the liver damage in the single NHP that suffered acute liver failure and shock (5). Liver failure and coagulopathy, with associated circulatory shock, have also been observed following the systemic administration of a high dose level (1.1×10^{14} vg/kg) of onasemnogene abeparvovec in subjects with SMA (141) (Section 4.1.1 of this document).

The IV administration of AAV9 or AAV-PHP.eB vectors (both encoding green fluorescence protein) in juvenile/adult NHPs (n=10), at dose levels ranging from 1.0- 2.0×10^{14} vg/kg, resulted in severe coagulopathy requiring early euthanasia of two NHPs 3 days post-dose. The remaining eight NHPs displayed transient elevation of liver enzymes, thrombocytopenia, complement activation and increased coagulation times at 3 days, with resolution by 7 days, post-dose. According to the authors, these data suggest that the severity of the liver findings and coagulopathy were dose-dependent and were higher in animals administered the AAV-PHP.eB vector. Furthermore, the observed toxicity was mitigated but not prevented by prophylactic steroid administration (prednisolone at 1 mg/kg/day starting one day prior to AAV vector administration) in one NHP administered 1×10^{14} vg/kg AAV-PHP.eB. The transient activation of the alternative complement pathway at 3 days post-dose was observed to coincide with thrombocytopenia and, according to the authors, was likely a contributing factor to acute liver injury in these animals (56). These data are briefly summarized in Section 5.3 of this document as they may also be relevant to recent findings of TMA in humans administered AAV vectors (as described in Section 5.1 of this document).

4.3.2 Hemophilia

Different serotypes of recombinant AAV encoding for clotting factors intended to treat hemophilia A or B have been investigated in rodent and nonrodent species, including mice, rats, ,

dogs, and NHPs (7, 161, 162). In an 18-week Good Laboratory Practice (GLP) toxicology and biodistribution study conducted in WT male mice, the administration of self-complementary AAV8 encoding FIX R338L at dose levels up to 4.0×10^{12} vg/kg did not result in adverse changes to liver enzyme levels or liver histology (161). Most dogs with hemophilia A administered AAV8 or AAV9 encoding canine FVIII via the portal or peripheral vein at dose levels, ranging from 1.2×10^{13} to 4×10^{13} vg/kg, displayed ALT levels within normal limits through 7-10 years post-dose. The ALT levels were transiently elevated in three of nine dogs at various timepoints (7). Additionally, IV administration of AAV8 encoding human FVIII (4×10^{12} particles/kg) to hemophilia A dogs that had previously received AAV8 encoding the canine Factor VIII, resulted in persistent elevation of ALT levels in one of the two dogs through 45 days post-dose, but no other biochemical parameters were perturbed (163). In 2 year-old male NHPs, mild transient elevations in liver enzymes (ALT, AST) were observed following IV administration of AAV5 encoding human FIX at dose levels up to 5×10^{12} vg/kg through 26 weeks (162). Findings of liver enzyme elevation observed in these animals were similar to findings in clinical studies (Section 4.1.2 of this document).

4.3.3 XLMTM

No adverse changes in liver enzyme levels or liver histology were reported out to 1 year post-dose in 10 week-old dogs with XLMTM that received AAV8 encoding for canine MTM1 (cMTM1) at dose levels of 4×10^{11} vg/kg (IV route) or 4×10^{11} vg/animal (IM route) (164). XLMTM dogs and normal littermates that were IV administered AAV8-cMTM1 up to a dose level of 5×10^{14} vg/kg did not show any adverse changes in liver enzymes (165). Additionally, no toxicity was reported in myotubularin1 (*Mtm1*)-knockout mice or WT littermates that were IV administered 3×10^{13} vg/kg AAV8-Mtm1 (164). In contrast, the administration of 3.0×10^{14} vg/kg AT132 (AAV8 encoding for human *MTM1* gene) in the ASPIRO clinical trial (Section 4.1.3 of this document) resulted in the 3 deaths associated with cholestatic liver failure, sepsis, or gastrointestinal bleeding.

4.4 **Advisory Committee DRAFT Discussion Questions**

1. Please discuss the merits and limitations of animal studies to characterize the risk of hepatotoxicity in human subjects and provide recommendations on preclinical study design elements, such as animal species/disease model and endpoints.

2. How should patients be screened and categorized based on their risk for developing liver injury, before AAV vector administration? Please discuss whether pre-existing hepatic conditions may predict the risk of serious liver injury.

3. What additional strategies could be implemented before or after AAV vector administration to prevent or mitigate the risk of liver injury?

4. What factors (e.g., level of disease severity) other than weight should be considered to determine the vector dose for systemic administration?

5. Considering the risk of toxicities observed in clinical trials with high doses of AAV vectors,
    a. please discuss whether an upper limit should be set for the total vector genome dose per subject.
    b. given that many AAV products contain significant amounts of empty capsids, please discuss whether an upper limit should be set on the total capsid dose.

5 Thrombotic Microangiopathy (TMA)

TMA is characterized by arteriole and capillary endothelial pathology and microvascular thrombosis. TMA presents clinically as a syndrome of hemolytic anemia, thrombocytopenia, and acute kidney injury. Two primary forms of TMAs manifest as thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS). Both conditions are rare. TTP occurs in approximately 3 per 1,000,000 adults. The incidence of HUS in children is estimated to be about 3 per 100,000. TMA is a hematologic emergency that requires prompt treatment. TTP is postulated to result from a severe deficiency of the enzyme ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13), due to acquired autoantibodies or, rarely, genetic mutations. The most common form of HUS (typical HUS) follows a diarrheal illness caused by Shiga toxin–producing *Escherichia coli*. Atypical HUS (aHUS) is considered to be associated with abnormal host susceptibility to complement-mediated damage. A variety of conditions such as malignancy, autoimmune diseases, drugs, toxins, and infections such as influenza, HIV, Epstein–Barr virus, or parvovirus may trigger an episode of TMA (166).

5.1 TMA Observed in Humans

Several cases of TMA have been reported in patients or study subjects who received AAV vectors.

5.1.1 SMA

In a recent publication, three patients with SMA developed TMA approximately 1 week after treatment with onasemnogene abeparvovec. All three had treatment with prednisolone 1 mg/kg/day for 30 days, as recommended in the onasemnogene abeparvovec PI. Two of these three patients had previous exposure to nusinersen; two had vomiting at presentation, and two had infection with encapsulated organisms. During their presentation, all three had laboratory evidence of complement activation (141). Standard treatments for TMA, such as transfusion (PRBC, platelets), glucocorticoids, and plasmapheresis resulted in recovery within 2-4 weeks.

However, one patient developed persistent hypertension; another patient developed hypertension and nephrotic syndrome, which resolved 3 months after initial treatment.

The onasemnogene abeparvovec PI was recently updated to include TMA in the "Warnings and Precautions" Section. The manufacturer also issued a "Dear Doctor Letter" to warn health care providers of the risks of TMA.

5.1.2 Duchenne Muscular Dystrophy (DMD)

Duchenne muscular dystrophy is an X-linked, recessive neuromuscular disorder caused by mutations in the *dystrophin* gene, affecting approximately 1 in 3500 male births worldwide. It is usually diagnosed between three and six years of age. These mutations disrupt the messenger ribonucleic acid (mRNA) reading frame, leading to the absence or near-absence of dystrophin protein in muscle cells. Dystrophin plays an important role in maintaining the structural integrity of the muscle cell, cushioning it from the stress and strain of repeated contraction and relaxation. Absence of dystrophin leads to muscle damage, with replacement by fat and collagen. With progressive degeneration of skeletal muscle (including breathing muscles) and cardiac muscle, there is loss of physical function in childhood and adolescence, with premature death from respiratory and/or cardiac failure in the second to fourth decade of life (167).

In DMD clinical studies, there have been reports of aHUS-like acute kidney injury, decreased platelets and complement activation, which were treated with hemodialysis and platelet transfusion and eculizumab (168). Proposed risk mitigation plans included: prophylactic use of eculizumab and C1 esterase inhibitor, increased prednisone dose in the month after AAV vector administration, and modification of the manufacturing process to decrease the percentage of empty capsid (169).

5.2 Potential Mechanisms of TMA

In recent studies, TMA following systemic administration of high AAV vector doses has been linked to complement activation (141). The exact mechanism and predisposing factors for

complement activation associated with AAV vector-induced TMA are not clear. To mitigate the risk of TMA, in some clinical studies, complement inhibitors such as eculizumab have been used prophylactically. Eculizumab (Solaris) is a monoclonal antibody that specifically binds to the complement protein C5 with high affinity and is indicated for treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH) and aHUS. However, it is unclear whether pre-treatment with complement inhibitors, such as eculizumab, can play a role in preventing AAV vector-mediated TMA (170).

5.3 Findings in Animal Studies

Limited data from published studies report similar toxicities in animals as those observed in clinical studies following administration of high doses of AAV vectors. In toxicology studies conducted in WT neonatal FVB/NJ mice using onasemnogene abeparvovec at doses up to 3.91×10^{14} vg/kg, changes in clinical chemistry suggesting decreased glomerular filtration rate, changes in hematology (e.g., increases in white blood cells, monocytes, and lymphocytes), and a mild to marked decrease in platelet count without a clear dose-response, were observed, in addition to hepatotoxicities (as described in Section 4.3.1.1 of this document) (171). In one published study, IV administration of AAVhu68-hSMN at 2×10^{14} vg/kg in three healthy juvenile NHPs resulted in acute systemic inflammation requiring euthanasia of one animal and elevated transient ALT and thrombocytopenia by Day 5 in two others (5). Lesions in the liver were also observed by Day 28 in these NHPs but no significant findings were reported in the kidneys. Similar findings of hepatotoxicity, thrombocytopenia and coagulopathy were also reported in a separate NHP study using IV administration of 7.5×10^{13} vg/kg of an AAV-PHP.eB vector (68).

In a more recent study (conducted by the same group), healthy NHPs administered 1×10^{14} or 2×10^{14} vg/kg of an AAV9 or AAV-PHP.eB demonstrated dose- and vector-dependent acute toxicities on Day 3 post-dose, including thrombocytopenia, liver enzyme elevation with or without hyperbilirubinemia and increased coagulation (56). Thrombocytopenia was coupled with activation of the alternative complement pathway, but not the classical complement pathway. There were no detectable IgM antibodies against the capsid on Day 3, and toxicities were largely resolved by Day 7. Prophylactic steroid use in this study did not prevent toxicities but appeared

to enable recovery. The authors highlight that acute presentation of thrombocytopenia and transaminase elevation has been observed in NHPs and humans but not in other species. While these findings should be considered within the context of differences in study designs (e.g., the use of different products, dose levels, concomitant therapies) and potential species-specific mechanisms of toxicity, there are shared similarities with the clinical findings of TMA, as described in Section 5.1 of this briefing document. Additionally, findings in NHPs may also be relevant to the incidence of hepatotoxicity in humans following AAV-vector administration, as described in Section 4.3.1.1 of this document.

5.4 **Advisory Committee DRAFT Discussion Questions**

1. Please discuss factors that may increase the risk of TMA following AAV vector administration.

2. Please provide recommendations on strategies that could be implemented before and after AAV vector administration to prevent or mitigate the risk of AAV vector-mediated TMA.

3. Considering the risk of toxicities observed in clinical trials with high doses of AAV vectors,
    a. please discuss whether an upper limit should be set for the total vector genome dose per subject.
    b. given that many AAV products contain significant amounts of empty capsids, please discuss whether an upper limit should be set on the total capsid dose.

6 Neurotoxicity: Dorsal Root Ganglion (DRG) Toxicities

6.1 Introduction

DRGs are bilateral structures located within or adjacent to the intervertebral foramina along the spine and are comprised of dense accumulations of primary sensory neuronal cell bodies, satellite glial cells, nerve fiber bundles, blood vessels, and immune cells (i.e., macrophages, T lymphocytes, and B lymphocytes) (172). Each DRG is surrounded by a thin layer of cerebrospinal fluid (CSF), yet also contains a unique, extensive network of arterioles and fenestrated capillaries, facilitating permeability between the blood and nervous tissues (173).

The primary sensory neurons (PSNs) located within each DRG are pseudo-unipolar in nature and project a single axon from their cell body, bifurcating at a T-junction. One branch of the T-junction innervates the peripheral tissues while the other branch extends and terminates in the dorsal horn of the spinal cord or traverses the length of the spinal column reaching the dorsal column nuclei in the brain stem (173, 174). The axons of the PSNs are bundled together and contain a mix of mechanosensory and nociceptive fibers that are responsible for transmitting sensory stimuli from the peripheral nerves to central terminals in the CNS.

6.2 Findings in Animal Studies

Several studies in NHPs evaluating multiple recombinant AAV vectors revealed histopathology findings in the PSNs of the DRG. These findings consisted primarily of minimal-to-moderate infiltration of mononuclear inflammatory cells, proliferating resident satellite cells, and PSN cell body degeneration within cervical, thoracic and lumbar DRG (6). Secondary to degeneration of neuronal cell bodies, minimal-to-moderate axonal degeneration (i.e., axonopathy) occurred along the ascending dorsal tracts of the cervical, thoracic, and lumbar sections of the spinal cord (SC) and in peripheral nerves. In these studies, the severity of the PSN pathology in the DRG peaks at approximately 1 month post-dose and remains stable out to 5 months post-dose, with a mild decrease in severity at 6 months post-dose (6).

The AAV-mediated histopathology findings in the DRG and SC have primarily been reported in NHPs. A few studies have also reported PSN pathology in young mini-Yucatan pigs (described later in this Section) and mice (5, 175, 176) following AAV vector administration. Available published data from mice have reported histopathology findings such as mononuclear cell infiltration, satellitosis, and multifocal neuronal necrosis in the DRG; however, data from comprehensive characterization of these findings are limited.

Several factors contribute to the incidence and severity of the AAV-mediated PSN pathology in the DRG. A meta-analysis of 33 preclinical studies in NHPs evaluated how various AAV vector components (i.e., capsid, promoter, and transgene), viral purification methods, dose levels, ROA, and the sex and age of animals affected the incidence and severity of the PSN histopathology findings (6). The authors identified certain capsids (i.e., AAV1), promoters (i.e., CAG), and transgenes (i.e., secreted) that resulted in a mild increase in the severity of the PSN pathology, while vector purification methods and the sex of the animals had a negligible impact. More importantly, this analysis revealed several factors that significantly contribute to the incidence and severity of the DRG histopathology findings in NHPs, including: 1) direct administration into the CSF via intra-cisterna magna (ICM) or intrathecal (IT) injection, and 2) administration of dose levels that are greater than 1×10^{13} vg/animal (approximately 1×10^{11} vg/g of brain weight) (5, 66, 177, 178).

The underlying cellular mechanisms that lead to the degeneration and axonopathy of the PSN following exposure to AAV gene therapies have not been fully characterized. However, evidence suggests that one mechanism is cellular stress due to high expression of transgene mRNA and/or proteins (6, 66). In support of this hypothesis, Hordeaux and colleagues demonstrated that reducing transgene expression in DRG neurons by including a specific microRNA target sequence prevented neuronal degeneration and axonopathy of the PSN following ICM administration of the vector (179). Additionally, the authors, as well as several other published studies, reported that co-administration of immunosuppressives was ineffective in reducing the severity and incidence of the AAV-mediated PSN pathology (66, 68, 178).

The AAV-mediated PSN pathologies observed in NHPs have been minimal-to-mild in severity and have not been associated with any clinical signs suggestive of neuropathic pain or ataxia following neurological examinations (6). Additionally, ataxia and/or tremors have been reported in three NHPs at 21 days post-dose following ICM/IT administration of an AAV vector encoding GFP (>1 × 10^{13} vg/animal); however, it was not clear if PSN pathology was observed (6). Proprioceptive deficits and ataxia have also been reported in three micro-Yucatan piglets 14 days post-dose following IV administration of an AAV vector encoding the *SMN* gene (2 × 10^{14} vg/kg) which correlated with severe neuronal degeneration in the DRG and axonopathy in the SC (179). These data suggest that a marked increase in the severity of the pathology in the PSN can result in neurosensory dysfunction; however, the clinical relevance and threshold at which this may occur are unknown.

6.3 DRG Toxicities in Humans

The clinical significance of AAV-mediated DRG toxicities in animal studies is not yet clear. To date, there have been two reports of neuronal loss within the DRG after AAV vector administration. A subject enrolled in the giant axonal neuropathy (GAN) study received an IT dose of 3.5 × 10^{13} vg of AAV9 GT. An autopsy performed 8 months later revealed severe neuronal loss in the absence of inflammation or clinical signs or symptoms of DRG toxicity (154). Furthermore, a subject enrolled in a clinical study for familial amyotrophic lateral sclerosis reported tingling sensations and pain in the hands and left foot 3-4 weeks after receiving 4.2 × 10^{14} vg of an AAV vector expressing superoxide dismutase 1 via IT route. Sural sensory-nerve action potentials and left median sensory potentials, which were normal before vector administration, were absent at 10 weeks. Additionally, bilateral superficial peroneal sensory nerve action potential amplitudes were reduced. An autopsy performed 15.6 months later revealed neuronal loss in the DRG (180).

6.4 **Advisory Committee DRAFT Discussion Questions**

1. Based on the published data, please discuss the relevance of the NHP cases of DRG and peripheral nerve toxicity to human subjects.

2. Please provide recommendations on preclinical study design elements, such as animal species/disease model, age, endpoints, and study duration, that may contribute to further characterization of these toxicities.

3. In addition to periodic neurological examinations, please provide recommendations on other methods to mitigate the risk of DRG toxicities in clinical studies.

7 Neurotoxicity: Brain Magnetic Resonance Imaging (MRI) Findings

7.1 Introduction

AAV GT are used to treat a variety of neurodegenerative disorders. One challenge for CNS-targeted AAV-based GT is delivery of therapeutic doses to cells/tissues affected by disease. CNS-targeted AAV vectors, such as AAV9 vectors are often administered IV. While IV delivery is relatively non-invasive, it is of poor targeting efficiency, mostly due to the limited ability of AAV vector to cross the BBB (181). Systemic delivery of CNS-targeted AAV vectors has often used the administration of weight-based high doses. The combination of systemic delivery and high doses may increase the risk of triggering anti-AAV and anti-transgene immune responses, and neuroinflammation (2).

Currently, the challenge of efficient delivery of AAV vectors to the CNS is being addressed by developing different serotypes/capsids of AAV fine-tuned to target specific sites in the CNS, although the clinical experience with such designed AAV vectors is limited. Another approach is by delivery of AAV vectors directly to specific brain regions via an ROA such as intraparenchymal injections using specific delivery devices. Direct vector administration to the CNS via intraparenchymal injection is thought to be more efficient than IV administration, achieving effective transduction near the injection site with comparatively lower vector doses (2). In a Phase 1 study, long-term follow-up of ten subjects with moderately advanced Parkinson's disease who received bilateral putamenal infusions of either a low or a high dose of AAV2-hAADC vector provided preliminary evidence of stable AADC expression for at least 4 years (182).

7.2 Brain MRI Findings in Humans

Several clinical trials are underway to deliver AAV GT to the brain, as listed in Appendix 1 of this document. AAV vectors are typically delivered with stereotactic guidance, to both sides of the brain parenchyma, through multiple burr holes, with 1-2 deposits per track, in a single neurosurgical session.

One study involved direct brain parenchymal administration of an AAVrh10 vector expressing tripeptidyl peptidase 1 (TTP1) for the treatment of late infantile Batten disease (183). Brain MRI within 48 hours after vector administration showed T2 hyperintensities [measured by T2-weighted imaging], diffusion hyperintensity [measured by diffusion-weighted imaging (DWI)], and restriction of diffusion [measured by apparent diffusion coefficient (ADC)] localized to the sites of AAV vector administration. During the course of the study, all thirteen subjects developed new T2 hyperintensity finding on brain MRI post product administration. Of these thirteen subjects, these localized abnormalities persisted in seven subjects at 18 months post-dose, while in two other subjects these abnormalities resolved. A brain MRI was not performed in the four other subjects at 18 months. Investigators noted no clinical sequelae attributable to these MRI findings, and reduced the dose by half a log for subsequent subjects. The dose reduction was based on the working hypothesis that the MRI findings localized to the vector administration sites represented mild persistent edema/inflammation in the areas at the tip of the catheter, where the highest concentration of the vector was deposited. In the acute period, SAEs, including seizures, abnormal movements, and emesis, occurred in six of thirteen children.

The MRI T2 hyperintensity signal raises concerns for the safety of intraparenchymal administration of AAV vectors. The cause of such findings is poorly understood. However, because this study was conducted in subjects with progressive neurodegenerative disorder using a non-randomized trial design, it may be difficult to discern whether clinical changes are caused by the AAV vector, the administration procedure (e.g., surgical procedure, delivery devices), or disease progression. Natural history studies have inherent drawbacks due to a lack of matching baseline characteristics, incomplete and missing data, multiple biases and confounders affecting the reporting and interpretation of adverse events. Studies with a concurrent control and appropriate blinding (as described above with AAVrh10 vector) would likely facilitate the interpretation of the brain MRI findings.

An additional challenge in interpreting safety signals is that some subjects (due to their age and developmental delays associated with some disorders) may not reliably report their symptoms

(e.g., numbness, paresthesia) or allow examiners to reliably assess neurological abnormalities during clinical examination (e.g., abnormality in language, memory, or fine motor skills).

7.3 Findings in Animal Studies

Studies conducted using AAV vectors demonstrated dose- and time-dependent histopathological findings and MRI abnormalities in the brains of rodents and nonrodent species following direct intraparenchymal administration. Select publications are summarized here. Sondhi et al. describe two studies conducted in healthy rats and NHPs using an AAVrh.10-based product carrying the human transgene CLN2 (AAVrh10hCLN2) (67). Animals in both studies were followed for 7 and 90 days post-dose. In rats, vector administered bilaterally into the striatum at a dose level of 1×10^{11} vg/animal resulted in perivascular cuffing (7/8 animals), gliosis (8/8 animals), and swollen microvesiculated neurons (8/8 animals) with moderate severity at the injection tracks on Day 90. Similar findings were also observed 3 mm caudal to the administration site in these animals but were not observed in control animals at any timepoint or in vector-administered animals evaluated at Day 7. This suggests a time-dependent response to the vector in rats.

In a second study, NHPs were administered a total of 180 µl (15 µl/injection site) of vector or phosphate buffered saline (PBS) bilaterally through 6 burr holes at 12 injection sites (8 caudal and 4 rostral sites in both the white and gray matter). Animals administered the vector developed moderate focal and linear gliosis and mild hemosiderin-laden macrophages at the injection tracks (caudal and rostral sections) on Day 7, which were not observed in the control animal. The severity and incidence of these findings were reduced by Day 90; however, mild to moderate white matter edema (spongiosis), accompanied by glial cells and focal perivascular cuffing of congested vessels by primarily lymphocytes were observed at the injection tracks (caudal and rostral). Because there was no concurrent control at this time point for comparison, it is unknown whether these findings would have also been observed with PBS administration. There were no significant changes in neurobehavioral outcomes in the NHPs. These data demonstrated the onset of possible vector-related histopathological findings at the injection tracks of rats and NHPs between Days 7 and 90 post-administration. Over time, the severity of some findings increased (in rats), while others decreased (in NHPs), and new abnormalities were evident. Because these

studies did not include the use of multiple dose levels, the corresponding impact of increasing concentrations of product in the brain could not be extrapolated. Similar histopathological findings were also reported using a different AAVrh.10h vector product (184).

More recently, long-term safety findings in the brain were reported following direct intraparenchymal administration of an AAVrh.10 vector carrying the human transgene for Arylsulfatase A (AAVrh.10hARSA) in healthy NHPs (178). In this study, animals were administered either PBS, 2.85×10^{10} or 1.5×10^{12} vg/animal of AAVrh10hARSA or 1.5×10^{12} vg/animal of AAVrh.10Null, a vector identical to AAVrh10hARSA with the exception of the expression cassette. Methods used for product administered were noted to be identical to those employed in the administration of AAVrh.10hCLN2 in humans (183). Animals were evaluated at Weeks 1, 13, 26 and 52. At 13 weeks, animals receiving 1.5×10^{12} vg/animal had histopathological findings of moderate to marked T (CD3+) and B (CD20+) cell infiltrates with prominent perivascular cuffing, and marked gliosis and microglial/macrophage (CD68+ cells) infiltrates at the injection tracks that corresponded with MRI abnormalities. These findings were not reversible by Week 52. Conversely, minimal to mild accumulation of T and B cell infiltrates were observed at the injection tracks of animals receiving the lower dose level of the vector; these findings were largely reversible by Week 52. Findings in control animals receiving PBS were characterized by mild to moderate microglial or macrophage infiltrates and minimal T cell infiltrates at the injection tracks. No B cells were observed at the injection tracks of these animals. Periodic videotaped behavioral assessments over 52 weeks did not demonstrate clinical sequelae of these findings at any vector dose level. Histopathological findings in the control group receiving 1.5×10^{12} vg/animal of AAVrh.10Null were similar to those in the high-dose level group (AAVrh10hARSA) but with a larger pathology area and loss of brain tissue, potentially attributable to higher levels of endotoxin in product lots. Animals administered AAVrh.10Null or high-dose AAVrhARSA also exhibited minimal to mild spinal cord pathology. These findings were not reversible by Week 52. Despite the presence of endotoxin in the AAVrh.10Null product lots, the authors hypothesize that the inflammatory response observed in animals administered AAVrh.10Null or high dose AAVrh10hARSA were due to the AAVrh10h capsid and not the transgene.

Published data using AAVrh.10 vectors indicate these findings were localized at or near the injection tracks, were dose-dependent, and had long-term persistence (67, 178, 184). Significant neurobehavioral deficits were not reported in association with these findings; however, the long-term impact on neurological function is unknown. The relationship between these findings in animals and findings in clinical studies (as summarized in Section 7.2 of this document) remains unclear. Additionally, these studies were conducted in healthy animals; it is unclear how histopathological and behavioral outcomes might manifest and progress in the presence of relevant neurological disease pathology.

7.4 **Advisory Committee DRAFT Discussion Questions**

1. Please provide recommendations for any preclinical neuropathological and behavioral assessments and duration of post-administration follow-up to identify and further characterize the risk of neurotoxicity following intraparenchymal administration of AAV vectors.

2. Please discuss the clinical significance, if any, of brain MRI abnormalities observed in clinical studies of AAV GT. Please discuss whether the delivery procedure vs. AAV vector may have contributed to the abnormal brain MRI findings.

3. Please provide recommendations on strategies that could be implemented before and after vector administration to prevent or mitigate the risk of CNS injury.

4. Please recommend a duration of monitoring for subjects who have abnormal brain MRI findings, or factors to consider for the determination of an appropriate duration of monitoring.

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9 Appendix 1: Current Central Nervous System (CNS) GT Studies

Disease	Trial Phase	Viral Vector	Serotype	Transgene	Delivery Route	Site of Delivery	Identifier*
Parkinson's disease	I	AAV	2	AADC	Parenchymal	Striatum	NCT03065192
Parkinson's disease	II	AAV	2	GAD	Parenchymal	STN	NCT00643890
Parkinson's disease	I	AAV	2	AADC	Parenchymal	Striatum	NCT01973543
Parkinson's disease	I	AAV	2	GDNF	Parenchymal	Putamen	NCT04167540
Parkinson's disease	I	AAV	2	GAD	Parenchymal	STN	NCT00195143
Parkinson's disease	I/II	AAV	2	AADC	Parenchymal	Putamen	NCT02418598
Parkinson's disease	I	AAV	2	GDNF	Parenchymal	Striatum	NCT01621581
Parkinson's disease	I	AAV	2	NTN	Parenchymal	Putamen	NCT00252850
Parkinson's disease	I	AAV	2	AADC	Parenchymal	Putamen	NCT01395641
Parkinson's disease	II	AAV	2	NTN	Parenchymal	Putamen	NCT00400634
Parkinson's disease	II	AAV	2	AADC	Parenchymal	Putamen	NCT03562494
Parkinson's disease	I/II	AAV	9	GBA	Parenchymal	Cisternal	NCT04127578
Parkinson's disease	I/II	AAV	2	NTN	Parenchymal	Putamen/SN	NCT00985517
Parkinson's disease	I	AAV	2	AADC	Parenchymal	Striatum	NCT00229736
Mucopolysaccharidosis IIIA	II/III	AAV	rh10	SGSH	Parenchymal	White matter	NCT03612869
Mucopolysaccharidosis IIIA	I/II	AAV	rh10	SGSH	Parenchymal	White matter	NCT01474343
Mucopolysaccharidosis IIIB	I/II	AAV	5	NAGLU	Parenchymal	White matter	NCT03300453
MLD/ALD	I/II	AAV	rh10	ARSA	Parenchymal	White matter	NCT01801709
Batten's disease	I	AAV	2	CLN2	Parenchymal	White matter	NCT00151216
Batten's disease	I	AAV	rh10	CLN2	Parenchymal	White matter	NCT01161576
Batten's disease	I/II	AAV	rh10	CLN2	Parenchymal	White matter	NCT01414985
Huntington's disease	I/II	AAV	5	Huntingtin	Parenchymal	Striatum	NCT04120493
Alzheimer's disease	I	AAV	2	NGF	Parenchymal	Nucleus basalis	NCT00087789
Alzheimer's disease	II	AAV	2	NGF	Parenchymal	Nucleus basalis	NCT00876863
Alzheimer's disease	I	AAV	rh10	APOE2	Parenchymal	Cisternal	NCT03634007
AADC deficiency	I	AAV	2	AADC	Parenchymal	SN/VTA	NCT02852213
AADC deficiency	I	AAV	2	AADC	Parenchymal	Putamen	NCT02926066
Giant axonal neuropathy	I	AAV	9	Gigaxonin	Intrathecal	CSF	NCT02362438
CLN6 disease	I/III	AAV	9	CLN6	Intrathecal	CSF	NCT02725580
Mucopolysaccharidosis II	I/II	AAV	9	IDS	Intrathecal	CSF	NCT03566043
CLN3 disease	I/II	AAV	9	CLN3	Intrathecal	CSF	NCT03770572
Alzheimer's disease	I	AAV	2	TRAIL	Intrathecal/ ventricular	CSF	NCT04133454
Duchenne muscular dystrophy	I/II	AAV	9	Micro-dystrophin	Intravenous	Systemic	NCT03368742
Duchenne muscular dystrophy	I/II	AAV	rh74	GALGT2	Intravenous	Systemic	NCT03333590
Duchenne muscular dystrophy	I	AAV	9	Mini-dystrophin	Intravenous	Systemic	NCT03362502
Duchenne muscular dystrophy	I/II	AAV	rh74	Micro-dystrophin	Intravenous	Systemic	NCT03375164
Duchenne muscular dystrophy	I/II	AAV	9	ACCA	Intravenous	Systemic	NCT04240314
Fabry's disease	I/II	AAV	2/6	GLA	Intravenous	Systemic	NCT04046224
Fabry's disease	I/II	AAV	8	GLA	Intravenous	Systemic	NCT04040049
Mucopolysaccharidosis II	I/II	AAV	2/6	IDS	Intravenous	Systemic	NCT03041324
Mucopolysaccharidosis IIIA	I/II	AAV	9	SGSH	Intravenous	Systemic	NCT02716246
Mucopolysaccharidosis IIIA	I/II	AAV	9	SGSH	Intravenous	Systemic	NCT04088734
Mucopolysaccharidosis IIIB	I/II	AAV	9	NAGLU	Intravenous	Systemic	NCT03315182
Mucopolysaccharidosis I	I/II	AAV	2/6	IDUA	Intravenous	Systemic	NCT02702115

Disease	Trial Phase	Viral Vector	Serotype	Transgene	Delivery Route	Site of Delivery	Identifier*
Mucopolysaccharidosis I	I	AAV	9	IDUA	Intravenous	Systemic	NCT03580083
Pompe's disease	I/II	AAV	8	GAA	Intravenous	Systemic	NCT04174105
Pompe's disease	I/II	AAV	NA	GAA	Intravenous	Systemic	NCT04093349
Spinal muscular atrophy 2	III	AAV	9	SMN	Intravenous	Systemic	NCT03505099
Spinal muscular atrophy 1	I/II	AAV	9	SMN	Intravenous	Systemic	NCT02122952
Spinal muscular atrophy 1	III	AAV	9	SMN	Intravenous	Systemic	NCT03306277
GM1 gangliosidosis (type II)	I/II	AAV	9	GLB-1	Intravenous	Systemic	NCT03952637
Becker muscular dystrophy	I	AAV	1	FS344	Intramuscular	Muscle	NCT01519349
Duchenne muscular dystrophy	I/II	AAV	1	FS344	Intramuscular	Muscle	NCT02354781
Duchenne muscular dystrophy	I	AAV	2	Mini-dystrophin	Intramuscular	Muscle	NCT00428935
Limb-girdle muscular dystrophy	I	AAV	1	SGCA	Intramuscular	Muscle	NCT00494195
Limb-girdle muscular dystrophy	I	AAV	1	SGCG	Intramuscular	Muscle	NCT01344798
Limb-girdle muscular dystrophy	I/II	AAV	rh74	SGCA	Intramuscular	Muscle	NCT01976091
Limb-girdle muscular dystrophy	I	AAV	rh74	Micro-dystrophin	Intramuscular	Muscle	NCT02376816
Pompe's disease	I/II	AAV	1	GAA	Intramuscular	Muscle	NCT00976352
Dysferlinopathies	I	AAV	rh74	DYSF	Intramuscular	Muscle	NCT02710500
Alzheimer's disease	I	ML	NA	NGF	Parenchymal	Nucleus basalis	NCT00017940
Parkinson's disease	I/II	LV	NA	ProSavin	Parenchymal	Striatum	NCT00627588
X-linked ALD	NA	LV	NA	ABCD1	Parenchymal	NA	NCT03727555
MLD/ALD	I/II	LV	NA	ARSA/ABCD1	Intravenous	Systemic	NCT02559830
MLD	II	LV	NA	ARSA	Intravenous	Systemic	NCT03392987
Fabry's disease	I/II	LV	NA	GLA	Intravenous	Systemic	NCT03454893
Mucopolysaccharidosis IIIA	I/II	LV	NA	SGSH	Intravenous	Systemic	NCT04201405
Mucopolysaccharidosis II	I/II	Lym	NA	IDS	Intravenous	Systemic	NCT00004454

ABCD1 = ATP-binding cassette transporter; ACCA = acetyl-CoA carboxylase alpha; ALD = adrenoleukodystrophy; ARSA = arylsulfatase A; ASPA = aspartoacylase; CLN2 = late infantile neuronal ceroid lipofuscinosis type 2; CLN3 = CLN type 3; CLN6 = CLN type 6; DYSF = dysferlin; FS344 = follistatin; GAA = glucosidase alpha; GBA = glucosylceramidase beta; GLA = galactosidase alpha; GLB-1 = galactosidase beta 1; IDS = iduronate 2-sulfatase; IDUA = alpha-1-iduronidase; LV = lentivirus; Lym = lymphocytes; ML = Maloney leukemia; MLD = metachromatic leukodystrophy; NA = not applicable; NAGLU = *N*-acetyl-alpha-glucosaminidase; NGF = nerve growth factor; NTN = neurturin; ProSavin = encodes tyrosine hydroxylase, AADC, and GTP-cyclohydrolase 1; SGCA = sarcoglycan alpha; SGCG = sarcoglycan gamma; SGSH = *N*-sulfoglucosamine sulfohydrolase; SMN = survival motor neuron; SN = substantia nigra; TRAIL = tumor necrosis factor–related apoptosis-inducing ligand; VTA = ventral tegmental area.

Source: Lonser RR et al. 2021. Direct convective delivery of adeno-associated virus gene therapy for treatment of neurological disorders. J Neurosurg. 134: 1751-1763 (185).