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Regulatory Insights

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REGULATORY PERSPECTIVE

Current global regulatory landscape for biodistribution & shedding assessment of rAAV gene therapies & recommendations of the IMI ARDAT consortium on future directions

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An understanding of the biodistribution and shedding profile of a gene therapy product following *in vivo* administration is an important element of the development program. Recommendations for biodistribution studies have been issued by various regulatory authorities with the most recent draft International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use guideline S12 'Nonclinical biodistribution considerations for gene therapy products' released for public comment on 3 June 2021. In this paper the Innovative Medicines Initiative, Accelerating Research & Development for Advanced Therapies consortium provides an overview of the current regulatory landscape for conducting shedding and biodistribution studies and makes a call for harmonization across regions. In addition, over the last three decades, a significant body of literature on biodistribution and shedding of AAV-based gene therapies has amassed, and we describe herein the initial stages of construction of a formal database of published biodistribution

and shedding data. The outputs from the database could be leveraged by Sponsors of AAV programs in regulatory submissions. This would reduce the need for unnecessary duplicative studies, streamline nonclinical development and expedite the arrival of this important class of novel medicines into clinic.

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Advanced therapies represent an important class of medicinal products where recent clinical successes have translated into large increases in the number of clinical trials, and investments in the field. In recent years there has been an increasing call to action for a more harmonized approach to requirements for developing gene therapies both among EU member states and between global regulatory agencies [1,2]. One such example of a move toward harmonization is the 2021 release of the draft International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guideline S12 "*Nonclinical biodistribution considerations for gene therapy products*" [3]. This draft guideline provides recommendations for the overall design of nonclinical biodistribution assessments, while also offering considerations for the interpretation and application of biodistribution data to support a nonclinical development program and clinical trial design. Whilst this is a welcome step toward harmonization, it is recognized that there are further opportunities to leverage existing data to reduce animal usage [4]. In general, scientific practice has shown that a lack of agreement on concepts, practices, standardized terms and definitions can hamper collaboration and alignment [5]. The Innovative Medicines Initiative (IMI; imi-europe.org), ARDAT (Accelerating Research & Development for Advanced Therapies) consortia was formed in early 2020 to fund five years of intensive research into AAV biology in the hopes that lessons learned will facilitate the development of AAV therapies. Over the last three decades, a significant body of research has been generated as developers of AAV gene therapies and academic researchers publish data from biodistribution and shedding studies. ARDAT proposes that, for AAV serotypes where biodistribution and shedding data is publicly available, regulatory applications could utilize the plethora of published literature instead of duplicating nonclinical studies. However, leveraging published data should also take into consideration the biological relevance of the animal species used to investigate biodistribution and/or shedding to the investigational product (e.g., virus-host interaction and tropism). In addition, assessments of transgene expression should consider the nature of the promoter, including tissue-specificity.

Our ongoing work to build the first formally constructed database of published biodistribution and shedding data on AAVs has revealed inconsistencies across the field in many aspects regarding the reporting of data (e.g., terminologies used, units of measurement), experimental design (analysis timepoints and tissue types analysed) and analytical methods used (e.g., lack of detailed information on method protocols, validation and detection limits). These inconsistencies could potentially limit the extrapolation of the data obtained from such studies to support regulatory submissions of vectors based on the same, or similar, serotype. The identification of these inconsistencies in reporting biodistribution and shedding data during database construction will facilitate future proposals for minimum data standards (including minimum analytical method validation) in this area.

The creation of a publicly available database of AAV biodistribution and shedding data

aims to enhance regulatory convergence and facilitate nonclinical development of AAV-based therapies. By allowing developers of such therapies to focus nonclinical studies to only product-specific issues, animal use with the associated time and investment costs will be reduced. In this way, it is hoped that the ARDAT biodistribution and shedding database will accelerate the development of these important therapies so that they become available as soon as possible to those patients who are waiting for the potentially life-changing benefits they offer.

REGULATORY AGENCY EXPECTATIONS FOR BIODISTRIBUTION

Biodistribution, as defined in the draft ICH Guideline S12, is the *in vivo* distribution, persistence, and clearance of a gene therapy product at the site of administration and in target and non-target tissues (including blood, cerebrospinal fluid and vitreous fluid), in biologically relevant animal species [3]. Data on biodistribution for a gene therapy medicinal product (GTMP) collected during nonclinical development will contribute to the design of nonclinical safety studies and can also inform dose decision, dosing schedules and monitoring plan for subsequent early-phase clinical trials.

The current regulatory guidelines discussing biodistribution requirements are summarized in Table 1. These clearly state that the biodistribution profile should be determined for a gene therapy product that has not previously been administered to humans and is proposed for a first-in-human (FIH) clinical trial. Under certain circumstances, biodistribution studies may also be conducted during later-phase clinical trials. For biodistribution studies, as well as nonclinical studies in general, incorporation of the 3Rs principles (reduce/refine/replace) regarding animal use [6] are recommended to eliminate the conduct of redundant studies.

In 2015, regulators from the International Pharmaceutical Regulators Programme (IPRP) Gene Therapy Working Group presented the expectations of various international regulatory authorities for nonclinical biodistribution studies [1]. Briefly, the importance of collecting biodistribution information early in product development to guide design of nonclinical toxicology studies and inform decisions on the need for additional nonclinical studies was emphasized. Specific requirements for biodistribution study design were discussed, including the use of a dosing protocol that mimics the proposed clinical protocol with appropriate safety margins (usually highest dose), assessment of all relevant organs, and extension of the analysis interval until the gene therapy product is not detected or a plateau phase is reached. Further considerations were made on inclusion of a relevant animal species and the use of different detection methodologies, including quantitative polymerase chain reaction (qPCR), immunohistochemistry, *in situ* hybridization, fluorescent protein expression, or *in vivo* imaging. The importance of collecting biodistribution data for new gene therapy vectors was discussed.

It was also acknowledged that nonclinical biodistribution studies have some limitations, such as the inherent differences between animals and humans (e.g., differences in organ size, receptor distribution, and physiology/pathophysiology) and that appropriate justifications for the choice of animal species/model would be required on a product-specific basis.

Importantly, there was also a recognition by some regulators that the use of shared or existing biodistribution data could facilitate development of gene therapy products of the same vector class by reducing or eliminating redundant nonclinical studies. However, the relevance of existing data should be justified on a case-by-case basis taking into consideration vector design, manufacturing process, dose, route of administration and disease. Furthermore, in the context of integration and germline transmission, a need for regulatory harmonization in approaches to study design and assessment was suggested.

A summary of biodistribution data submitted to support EMA and FDA marketing

TABLE 1

Summary of global biodistribution guidelines[1].

Health Authority	Guideline	Summary of recommendations on biodistribution
European Medicines Agency (EMA)	Guideline on quality, nonclinical and clinical requirements for investigational advanced therapy medicinal products [7]	▶ Data should be available to provide information on the persistence, duration of effect, and target organs to support the design and duration of safety study(ies). ▶ Extrapolation might be possible case by case with exemption to replication competent viral vectors where nonclinical biodistribution studies are expected prior first clinical trial.
	Guideline on the nonclinical studies required before first clinical use of gene therapy medicinal products [8]	▶ Studies should provide data on all organs, whether target or not. ▶ Observation time should cover persistence of signal (i.e., duration of transgene expression and activity) and include time-points for which there is no signal detection, if applicable. ▶ The dosing should mimic the clinical use with appropriate safety margins.
	Guideline on nonclinical testing for inadvertent germline transmission of gene transfer vectors [9]	▶ The biodistribution studies should be performed using the final vector construct with the gene of interest, with two dose levels at minimum, in at least two species, one of which should be a non-rodent species. The study should be conducted using both sexes. If no positive and persistent signal in gonads is detected in biodistribution studies, this might exclude the need for further nonclinical germline transmission studies. ▶ As a worst-case scenario, biodistribution studies should also be carried out using the intravenous route of administration with a dose per kg body weight at least 10-fold higher than the one to be administered to subjects/patients.
	Guideline on the risk-based approach according to annex I, part IV of Directive 2001/83/EC applied to Advanced Therapy Medicinal Products [10]	▶ Biodistribution data is identified as important for the identification/evaluation of risk factor-risk relationships translated later into support for MAA.
	Guideline on scientific requirements for the environmental risk assessment of gene therapy medicinal products [11]	▶ Nonclinical biodistribution and shedding data can be used to define which tissue samples are to be taken and the timing of sampling pre- and post-administration. For example, urine, faeces or mucosal nasal swabs, could be analysed as a part of a biodistribution study for the presence of the GMO.
	Reflection paper on quality, nonclinical and clinical issues related to the development of recombinant adeno-associated viral vectors [12]	▶ The guidance states that non-clinical biodistribution data of a human serotype-derived vector in animal models may not correlate with human biodistribution and there may be a scientific justification in some cases for the use of serotypes specific to the animal model used. ▶ Transgene expression should also be investigated. ▶ Study design should include where possible assays for the detection of co-packaged plasmid DNA to assess distribution and persistence. ▶ Impact of concomitant treatments (e.g., immuno-suppression) on biodistribution should also be considered. ▶ Germline transmission studies should be conducted before first administration to humans.
US Food and Drug Administration (FDA)	Guidance for Industry Pre-clinical Assessment of Investigational Cellular and Gene Therapy Products [13]	▶ The characterization of vector presence, persistence, and clearance profile can inform the selection of the GT product dosing schedule, the monitoring schedule for various activity/safety parameters, and the animal sacrifice time points in the definitive pre-clinical studies. ▶ Biodistribution data, coupled with other pre-clinical safety endpoints such as clinical pathology and histopathology, help determine whether vector presence or gene expression correlates with any tissue-specific detrimental effects in the animals.
	Guidance for Industry Long Term Follow-Up After Administration of Human Gene Therapy Products [14]	▶ Nonclinical studies to assess persistence are recommended to inform the potential risk of delayed adverse events and to aid in planning for long-term follow up in clinical studies ▶ To determine vector persistence, PCR assay is recommended – persistence is indicated by the presence of detectable levels of vector sequences above a threshold level (≥ 50 copies/µg genomic DNA) and the absence of a downward trend over several timepoints. ▶ Nonclinical data from similar gene therapy products may be used to support conclusions with regard to persistence (e.g., a vector with identical route of administration and final formulation that only differs in the coding sequence for the proposed therapeutic gene product) ▶ Biodistribution studies can be conducted as 'stand-alone' or as part of pharmacology or toxicology studies. ▶ Detailed recommendations for animal study design are provided, which include considerations regarding the use of final product formulation, number of animals required of each sex per timepoint, use of intended clinical route of administration, dose levels, characterization of product distribution and clearance kinetics. ▶ A minimum panel of tissues to be sampled in biodistribution studies is recommended along with general considerations for tissue collection. ▶ Recommendations for qPCR assays are made including demonstrated limit of quantitation and use of spike controls to determine assay sensitivity.
PMDA	Quality and Safety Assurance for Gene Therapy Products and Human Cell-based Products, 9 July, 2019 [15]	▶ Biodistribution of the gene therapy product or human cell-based product in suitable animals should be presented as basic data for evaluating the safety and efficacy of the product. Analysis of biodistribution can clarify distribution not only to the intended tissues but also to non-target tissues and germ cells, making it possible to identify which organs to focus on when evaluating safety and the risk of inadvertent integration in humans. ▶ Clarifying aspects of vector persistence such as distribution and elimination will yield information on suitable timing for analysis in humans. ▶ Biodistribution data may be useful when considering the toxicological significance of abnormal findings specific to certain tissues in toxicity studies. ▶ If biodistribution studies are not performed before starting clinical trials of a new gene therapy product or human cell-based product, the reason for this must be explained. ▶ When analysing biodistribution, tissue, blood and other materials should be collected at defined intervals after administration of the gene therapy product or human cell-based product, and the vector copy number should be measured using qPCR or similar methods. In addition, measuring changes in the vector copy number over time will yield information on the fate of the vector. ▶ If expression constructs are found in specific tissues, bodily fluids, etc., expression of the target gene from these expression constructs should be analysed.
IPRP[2]	Reflection paper – "Expectations for Biodistribution (BD) Assessments for Gene Therapy (GT) Products" [16]	▶ Overarching focus on the need, design, conduct and analysis of gene therapy biodistribution studies. Details are provided for study design. Suggestions on implementation of data into design of FIH clinical trials. Considerations on when additional biodistribution studies are needed.

[1] Note: ICH guideline S12 "Nonclinical biodistribution considerations for gene therapy products" was released for public comment on 3 June 2021, but has not been included in the table as the recommendations may change in the final document.
[2] The International Pharmaceutical Regulators Programme (IPRP) is a consortium of international regulators from Australia, Brazil, Canada, China, European Union, India, Japan, Singapore, South Korea, Switzerland, Thailand and United States
EMA: European Medicines Agency; FDA: Food and Drug Administration (United States); GMO: Genetically modified organism; GT: Gene therapy; IPRP: International Pharmaceutical Regulators Programme; MAA: Marketing Authorization Application; PMDA: Pharmaceuticals and Medical Device Agency (Japan); qPCR: Quantitative polymerase chain reaction.

authorizations of AAV-based GTMPs is provided in Table 2.

REGULATORY AGENCY EXPECTATIONS FOR SHEDDING

From the regulatory perspective, shedding is defined as the dissemination of virus/vector through secreta (e.g., urine, saliva, nasopharyngeal fluids), excreta (faeces) or through the skin (pustules, sores and wounds) of the patient [22,23]. The selection of sample types to be collected for shedding assessment are dependent on a variety of factors such as route of administration, virus tropism, and the natural route of transmission of the parental virus.

General regulatory expectations with regard to shedding data to support clinical trial applications (CTAs) and applications for marketing authorization in the EU, and investigational new drug applications (INDs) and biologics license applications (BLAs) in the US, are described in Table 3. Considerations for environment risk assessments are summarized in Table 4, highlighting relevant differences between the EU and US.

In the European Union, the EMA guideline "*Nonclinical Studies Required Prior to Clinical Use of Gene Therapy Medicinal Products*" states that an investigation of GTMP shedding is a component of the minimum requirements for nonclinical studies before first use in human subjects [8]. Furthermore, the incorporation of shedding studies in an animal model during nonclinical development is also recommended in the EMA guidance on "*Environmental Risk Assessment of Gene Therapy Medicinal Products*" [11] to support the environmental risk assessment (ERA) required for marketing authorization. Complying with GMO requirements at the time of CTA is complex, varies significantly across EU Member States and is leading to delays to clinical trials with ATMPs [2].

In the United States, an environmental assessment is not required at the start of clinical trials for investigational new drugs, except under special conditions. A full report of clinical shedding in the target patient population(s) is expected in the Biologics License Application (BLA) for a GTMP in order to address the potential for transmission to untreated individuals [23]. Clinical shedding reports should include a summary of nonclinical findings (if conducted), rationale for study design and assay development, details of the data collection/sampling plan, procedures for sample handling, collection and storage, description of assays, an analysis of shedding data, and an estimate of the potential for transmission to untreated individuals [23].

Of note, a process of regulatory harmonization with respect to shedding was initiated in 2009, as the topic "*Virus and Gene Therapy Vector Shedding and Transmission*" was the subject of an ICH concept paper [30] and an ICH Considerations document [24], which addressed the general principles to be considered when designing nonclinical and clinical shedding studies, including analytical methods, sampling profiles and schedules. The ICH Considerations document emphasized that data obtained from nonclinical studies of shedding can aid the design of clinical shedding studies by providing an estimation of the likelihood and extent of shedding in humans. 'Stand-alone' nonclinical studies of shedding are not required and shedding analyses can be incorporated into toxicity and/or biodistribution studies, for example by the analysis of urine, faeces or mucosal nasal swabs for the presence of GTMP [11,24]. However, two years later, the ICH steering committee concluded that harmonization on this topic could not be supported due to "*the current state of the science and related resource allocation*".

The extent of shedding data required by regulatory authorities to assess the potential risk to third persons or the environment is dependent on the biological properties of the viral vector. As summarized in Table 5, the biological properties of AAV vectors support the conclusion that they represent a very low shedding risk.

As summarized in Table 6, according to publicly available information, clinical

TABLE 2

Summary of biodistribution data submitted to support EMA marketing authorization of AAV-based GTMPs.

Product / Reference documents	Vector subtype (transgene) Posology / Route of administration	Therapeutic indication	Biodistribution data to support marketing authorization
Luxturna® (voretigene neparvovec) [17,18]	AAV2 (hRPE2) 1.5×10^{11} vg/eye Subretinal injection	Treatment of adult and paediatric patients with vision loss due to inherited retinal dystrophy caused by confirmed biallelic RPE65 mutations and who have sufficient viable retinal cells.	Biodistribution of Luxturna was evaluated at three months following subretinal administration in non-human primates. The highest levels of vector DNA sequences were detected in intraocular fluids (anterior chamber fluid and vitreous) of vector-injected eyes. Low levels of vector DNA sequences were detected in the optic nerve of the vector-injected eye, optic chiasm, spleen and liver, and sporadically in the stomach and lymph nodes. In one animal administered with Luxturna at 7.5×10^{11} vg (five-times the recommended per eye dose), vector DNA sequences were detected in colon, duodenum and trachea. Vector DNA sequences were not detected in gonads.
Zolgensma® (onasemnogene abeparvovec) [19,20]	scAAV9 (SMN1) 1.1×10^{14} vg/kg Intravenous infusion	Treatment of patients with 5q SMA with a bi-allelic mutation in the SMN1 gene and a clinical diagnosis of SMA Type 1 or patients with 5q SMA with a bi-allelic mutation in the SMN1 gene and up to 3 copies of the SMN2 gene	The biodistribution and SMN transgene expression profile of ZOLGENSMA were evaluated in neonatal FVB mice through 12 and 24 weeks. Following intravenous administration of 1.5×10^{14} vg/kg Zolgensma, the highest vector DNA concentration was detected in the heart, followed by the lung, liver, lumbar spinal cord, quadriceps muscle, brain, ovary, spleen, and testis. The human SMN mRNA transcripts had a similar tissue expression profile with highest levels in the heart, followed by quadriceps muscle, liver, lung, brain, and lumbar spinal cord. Low levels of SMN mRNA were detected in the spleen and gonadal tissues. Additionally, biodistribution was evaluated in two patients who died 5.7 months and 1.7 months after infusion of Zolgensma at the dose of 1.1×10^{14} vg/kg. Both cases showed that the highest levels of vector DNA were found in the liver, followed by the spleen, inguinal lymph node and heart. Vector DNA was also detected in the muscles, peripheral nerves, kidney, pancreas, lung, spinal cord, brain, and thymus. Immunostaining for SMN protein showed generalized SMN expression in spinal motor neurons, neuronal and glial cells of the brain, skeletal muscles, heart, liver, kidney, lung, pancreas, spleen, thymus, stomach, large intestines, small intestines, and inguinal lymph nodes.
Glybera (alipogene tiparvovec) [21]	AAV1 (human lipoprotein lipase gene) Intramuscular injections in the legs, 1.5×10^{12} vg per injection site	Familial lipoprotein lipase deficiency	Following intramuscular administration of Glybera to mice, vector DNA was transiently detected in the circulation. Eight days after administration, high levels of vector DNA sequence were detected in injected muscle and the draining lymph nodes. Except for the site of injection, the highest vector DNA copy numbers were found in the liver and blood. The lowest number of copies was found in the brain, lung, heart and non-injected groups of muscle. In gonads and reproductive organs, vector DNA copies were found at low levels. After time, residual vector DNA levels remained high in the injected muscle and inguinal lymph nodes while decreasing steadily in the other organs. The levels of Glybera vector DNA found in gonads were measurable but lower than in other non-target organs. Immunosuppressant co-treatment did not influence the biodistribution pattern neither at low dose nor at high dose in mice. The biodistribution pattern was very similar in the other tested species (cats and rabbits).

Note: [1] The marketing authorization for Glybera expired in 2017, following the decision of the marketing authorization holder not to apply for a renewal due to a lack of demand for the product.
AAV: Adeno-associated viruses; SMA: spinal muscular atrophy

shedding data was presented to support marketing authorization of both AAV-based GTMPs currently on the market; Zolgensma and Luxturna, as well as for the withdrawn product Glybera. It is noteworthy that nonclinical shedding data did not appear to be presented for any of the aforementioned products.

In the case of Zolgensma, the possible expression of transgene (or partial/nonvector related sequences arising as viral packaging impurities from the manufacturing process), induction of immune responses against capsid proteins, and vector mobilization were identified in the ERA as the potential hazards related to shedding and third party transmission. The likelihood of shedding was considered to be high, considering clinical trial data that showed that vector shedding occurred in urine, saliva and faeces. Due to the replication-deficient nature of the vector, no infectious particles were expected to be shed (although this was not formally shown in clinical studies), and therefore the environmental consequences of transmission to non-target individuals to occur were expected to be limited [20].

The presence of replication-competent AAV (rcAAV), arising due to either impurities in the manufacturing process or complementation by co-infection with wild-type AAV, was considered to pose a negligible risk. In particular, the limitations on packaging capacity for AAVs do not permit the therapeutic transgene (SMN1) and the genes required for helper virus-mediated replication (Rep and Cap) to exist in the same viral particle.

Shedding of vector particles was not specifically identified as a potential hazard in the ERA for Luxturna, due to the transient and low level of shedding reported in clinical studies [17].

The ERA for Glybera contained an assessment of the potential risks associated with third-party transmission to healthy persons due to the reported shedding of viral vector from patients through urine, faeces, saliva, and seminal fluid [21]. Several aspects were considered including; i) the effect of transgene over-expression, ii) possible non-site specific AAV integration and insertional mutagenesis, iii) possible adverse effects associated with certain vector elements (e.g., tumorigenicity risks associated with the Woodchuck hepatitis virus post-transcriptional regulatory element [WPRE] present in the viral genome), iv) presence of replication-competent AAV by recombination events occurring during manufacturing or after administration to patients, v) Incorporation of shed DNA by other animal or plant species; vi) Germline transmission. EMA and the national competent authorities responsible for GMO regulation agreed with the Applicant's conclusion that Glybera was a negligible risk to human health (of third parties) and the environment.

Given that the biological properties of AAVs suggest that the potential risk to third parties via shedding is generally low, ARDAT proposes that for serotypes where biodistribution data is publicly available, regulatory applications utilize the plethora of published literature instead of duplicating nonclinical AAV biodistribution studies. This is consistent with the observation that nonclinical shedding data did not appear to be presented at the time of Marketing Authorization Application for AAV-based medicinal products currently approved in the EU.

REGULATORY AGENCY EXPECTATIONS FOR BIOANALYTICAL METHODS

The current 'gold standard' for the measurement of specific DNA or RNA corresponding to vector genome or transgene expression products in tissues and biofluids is qPCR for DNA and quantitative reverse-transcriptase PCR (qRT-PCR) for RNA. These assays are used to assess both biodistribution and shedding. As stated in ICH S12, qPCR-based assays have the advantage that they are sensitive, reproducible, and rapid [3].

In (non)clinical biodistribution studies other techniques that can be used to quantitatively assess vector or expression product biodistribution include enzyme-linked immunosorbent assay (ELISA), digital droplet PCR, flow cytometry and other *in vivo* and *ex vivo* imaging techniques. Other techniques such as immunohistochemistry (IHC), Western blot, in situ hybridization (ISH) can be used for a qualitative assessment of transgene expression. A comprehensive description of the methodology and a justification for the

technique used should also include the performance parameters of the method [3].

There is also a recognition that the standardized requirements for bioanalytical method development and validation may be difficult to apply for these types of assays and in the absence of specific regulatory recommendations for qPCR / qRT-PCR method validation it remains a challenge for the field to develop standardized methods for the analysis of biodistribution and shedding [31]. A recently published article provides some orientation with regard to those aspects of qPCR-based method development and validation which should be considered; extraction efficiency of the AAV-based product in each matrix, inhibitory effects of the matrix on PCR components (e.g., DNA polymerase), and primer design and selection (e.g., including at least a portion of the inserted transgene to avoid false positives arising from the presence of wild-type AAV) [32].

While the guidance documents on bioanalytical method validation released by EMA, FDA and ICH are comprehensive, their focus is on the detection of small molecule drugs and therapeutic proteins by chromatographic and ligand-binding assays in a limited set of biological matrices such as serum, blood, plasma, and saliva [33–35]. There is no specific mention of nucleic acid amplification techniques such as qPCR in those documents.

The FDA draft Guidance for Industry on the Preclinical Assessment of Investigational Cellular and Gene Therapy Products states that qPCR assays should be used to determine the number of vector copies per microgram of genomic DNA in tissues/biological fluids, but there is no mention on whether validation is required [13]. Likewise, FDA does not require validation of qPCR assays used to assess shedding, only that the assay should be qualified to meet minimal performance capabilities (specificity, sensitivity, reproducibility, and accuracy) and be suitable for the intended purpose [23].

In contrast, EMA guidance states that the methods of analysis used in nonclinical studies should be validated with the test article in the appropriate matrix [36]. It is noteworthy that during the review of the MAA for Luxturna, the lack of validation of the assays used to detect the AAV-based viral vector to an acceptable standard meant that the nonclinical biodistribution data obtained was not considered definitive by the Agency [17]. This was not believed to be an issue in the FDA's Pharmacology-Toxicology Review for Luxturna, which stated that the report of the qPCR analysis for the evaluation of biodistribution and shedding in AAV2-hRPE65 studies was reviewed and deemed adequate by CMC reviewers.

While method validation is not explicitly mentioned in the newly released draft ICH guidance on nonclinical biodistribution studies, the establishment and documentation of the sensitivity and reproducibility of the quantification method is recommended [3]. The draft guidance also states that spike and recovery experiments are required to demonstrate the ability to detect target. Furthermore, the Gene Therapy Working Group of the International Pharmaceutical Regulators Programme also stated that method validation should be considered in the 2018 reflection paper "*Expectations for Biodistribution Assessments for Gene Therapy Products*" [16].

It should be noted that, to some extent, cross-validation of qPCR-based methods used for release characterization (e.g., viral particle quantification) may also be applicable in the context of bioanalytical methods used to detect viral genomes in biodistribution and shedding studies.

According to the ICH Considerations document on virus and vector shedding, PCR and infectivity are the two assays typically used for the detection of shed virus/vector. qPCR-based assays to detect viral genetic material are recommended. However, assays based on nucleic acid detection do not differentiate between intact (and potentially infectious) viral vector and non-infectious degraded or fragmented virus. Therefore, infectivity assays may be required for adequate assessment of the potential risk for transmission to third persons [24].

TABLE 3

Summary of general regulatory considerations for shedding.

Authority issuing guideline	Name / reference of guidance document	Summary of key considerations
ICH	General Principles to Address Virus and Vector Shedding [24]	**Quality** ▶ For replication-incompetent GTMPs, potential replication-competent recombinants that may arise during manufacturing should be characterized. **Nonclinical** ▶ Although shedding profiles obtained from nonclinical studies may not directly correlate with the human situation, the data obtained can be used to estimate the likelihood and extent of shedding in humans. ▶ Animal disease models may be most appropriate to assess shedding. ▶ 'Stand-alone' nonclinical shedding studies are not necessary, shedding endpoints can be incorporated in biodistribution and/or toxicology studies. ▶ A range of excreta and secreta samples can be collected, most commonly urine and faeces. To obtain sufficient sample size/volume, the pooling of samples collected from several animals receiving the same dose is suggested. **Clinical** ▶ The design of clinical shedding studies should consider the biological properties of the parental virus/vector, replication-competence of the product, dose, route of administration and patient population. ▶ The sampling schedule is usually more frequent in the initial period post-administration and should continue until multiple negative samples are obtained. ▶ The potential for a second round of shedding in the case of replication-competent vectors and the possible impact of immunosuppressive regimens should be taken into consideration when designing the study. ▶ The potential for transmission to third parties (e.g., family members and healthcare workers) should be evaluated if clinical shedding is observed. **Analytical methods** ▶ The use of qPCR to detect viral genetic material in shed samples is recommended and the use of an infectivity assay is considered important to accurately assess the potential for transmission of shed material. ▶ Assay interference from the biological matrix is an important consideration and sample dilution may be necessary.
EMA	Guideline on nonclinical studies required before first clinical use of gene therapy medicinal products [8]	Biodistribution studies should include an investigation of shedding as one of the minimal requirements for nonclinical studies on GTMPs before first use in human subjects.
	Guideline on Scientific Requirements for the Environmental Risk Assessment of Gene Therapy Medicinal Products [11]	▶ Shedding data from both nonclinical and clinical development (data from one or more clinical trials) may contribute to the ERA. ▶ Analysis of urine, faeces and mucosal swabs for the presence of the GMO are suggested, using sensitive and state-of-the-art methods. ▶ The presence of vector genome in shed samples is assumed to represent a potential for transmission into the environment. Assays to demonstrate non-infectivity of shed material should be as sensitive or more sensitive than the assay used to detect vector genome. ▶ Shedding of a GTMP in itself is not considered an adverse effect for the environment, but is rather a factor which is used in the evaluation of the likelihood of a particular environmental adverse effect – i.e., increased shedding resulting in higher environmental exposure only leads to a high risk if significant consequences have been identified (e.g., potential of transmission to third persons and/or other species).
	Guideline on Safety and Efficacy Follow-up – Risk Management of Advanced Therapy Medicinal Products [25]	Shedding data can be used to inform the preparation of the risk management plan and measures to mitigate the potential risk to close contacts of treated patients
	Guideline on Environmental Risk Assessments for Medicinal Products containing, or consisting of Genetically Modified Organisms [26]	The extent of shedding by target patients should be considered in the evaluation of the likelihood of an identified adverse environmental effect.
	Guideline on the Risk-based Approach According to Annex I, part IV of Directive 2001/83/EC applied to Advanced Therapy Medicinal Products [10]	Although shedding is not specifically mentioned, a stated risk associated with the clinical use of ATMPs was disease transmission (presumably to third parties) – as such, shedding data may be useful for determination of the relevance of a particular risk during risk identification. Risk identification can also be supported by reference to relevant literature data.
	Reflection paper on quality, nonclinical and clinical issues related to the development of recombinant AAV [12]	▶ Analysis of the shedding of co-packaged plasmid DNA sequences arising from the manufacturing process should be considered in nonclinical studies ▶ If vector DNA is detected in shed material (e.g., saliva, serum, urine and semen), ideally samples should be followed up for infectious virus quantification; data derived from nonclinical and early clinical studies can be used to assess the likelihood of transmission and to justify the extent of viral shedding evaluation in later clinical studies. ▶ All available data on viral shedding should be used in the ERA. ▶ In clinical studies, samples to be collected and timing of collection for shedding analysis should be justified on the basis of nonclinical data and/or the profile of the parental virus, practical feasibility and ethical justification of sampling. Examples of samples that could be collected include blood/serum, tears, urine, serum, buccal swabs/sputum, lung lavage and faeces.

AAV: Adeno-associated viruses; EMA: European Medicines Agency; ERA: Environment Risk Assessment; FDA: Food and Drug Administration (United States); GMO: Genetically Modified Organism; GTMP: Gene Therapy Medicinal Product; ICH: International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use; LOD: Limit of Detection; PMDA: Pharmaceuticals and Medical Devices Agency (Japan); qPCR: Quantitative Polymerase Chain Reaction.

TABLE 3 (CONT.)

Summary of general regulatory considerations for shedding.

Authority issuing guideline	Name / reference of guidance document	Summary of key considerations
FDA	Guidance for Industry – design and analysis of shedding studies for virus and bacteria-based gene therapy and oncolytic products [23]	**Nonclinical** ▶ Nonclinical data cannot substitute for human shedding studies on the basis that animals may not adequately predict the shedding profile in humans, particularly with respect to patient-specific factors such as differences in immune status at the time of product administration. ▶ Nonclinical shedding data may possibly be requested for replication-competent GTMPs in certain cases (e.g., no previous human exposure to product, changes in route of administration). ▶ Shedding data can be collected from nonclinical studies designed to assess safety or biodistribution. Such shedding data may inform the types of samples to be collected during clinical shedding studies. **Clinical** ▶ Recommendations for the timing of shedding data collection during different phases of clinical development are given; for replication-competent products shedding data should be collected from Phase I onwards, while for replication-incompetent or deficient products shedding data should be collected during Phases II and III of clinical development, after a dose and regimen have been determined. ▶ Modifications of the administration route, dose regimen and indication may cause alterations in shedding profile – shedding data from pivotal studies should be collected. ▶ Comprehensive advice on shedding study design and reporting is provided: ▶ Frequency of sample collection - sampling of shed material should begin immediately after product administration, irrespective of replication competence. Frequent sampling during the initial weeks post-administration is advised to ensure the shedding pattern is accurately captured. ▶ Duration of sample collection - Sampling should continue until three consecutive data points are obtained at or below the LOD of the shedding assay or if a decreasing trend reaches a plateau for at least three consecutive data points. Monitoring periods may need to be longer for patients who are immune-compromised or are receiving immunosuppressive regimens. ▶ Type(s) of samples collected – types of clinical samples collected to assess shedding are dependent on a range of factors including administration route, vector tropism, natural route of transmission and data obtained from nonclinical biodistribution/shedding studies. ▶ Sample storage – to minimize degradation of product-specific nucleic acids and to ensure no loss of product-specific infectivity appropriate storage conditions for different types of samples need to be established. ▶ Overall analysis of shedding data should address the potential for transmission to untreated individuals due to shedding (i.e., the nature of the shed material and the extent of shedding). ▶ Analytical Methods ▶ A quantitative assessment of shedding is recommended (e.g., number of genome copies or infectious units). qPCR is commonly used due to high sensitivity and practicality (e.g., ease of assay standardization). If product is replication-competent detection of nucleic acids should be followed up with infectivity or growth-based assays. ▶ The effect of different biological matrices on assay performance (including selectivity, specificity and sensitivity) should be well understood, particular in the case of shed samples rich in complex organic matter (e.g., urine and faeces).
PMDA	Quality and Safety Assurance for Gene Therapy Products and Human Cell-based Products [15]	The risk of human transmission to of GTMPs should be evaluated, including the risk of a vector administered to a patient being transmitted to a third party other than the patient with specific reference to the "ICH Consideration Document: General Principles to Address Virus and Vector Shedding" [24]

AAV: Adeno-associated viruses; EMA: European Medicines Agency; ERA: Environment Risk Assessment; FDA: Food and Drug Administration (United States); GMO: Genetically Modified Organism; GTMP: Gene Therapy Medicinal Product; ICH: International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use; LOD: Limit of Detection; PMDA: Pharmaceuticals and Medical Devices Agency (Japan); qPCR: Quantitative Polymerase Chain Reaction.

The general recommendation is for a 'step-wise' or 'tiered' approach, whereby the presence of viral genome is detected by qPCR in the first instance, followed by a suitable infectivity assay if viral DNA is detected above a certain threshold [23]. The ICH Considerations document suggests that an assessment of infectivity would be necessary if vector DNA in shed samples is detected at a level greater than the detection threshold of the infectivity assay [24]. Of note, nuclease treatment of clinical shedding samples followed by PCR amplification or amplification of the full-length viral genome from the intact virus particles has also been suggested by FDA as an alternative method for detection of infectious virus [23].

There is relatively little regulatory guidance with regard to infectivity assays. The ICH Considerations document includes an acknowledgement that assays to measure the extent of shedding have the additional complication that detection of infectious virus should be optimized in several different matrices, some of which are rich in complex organic matter (e.g., urine and faeces) and/or genomic material from organisms forming part of the body's natural flora (e.g., saliva and nasal swabs). These matrix effects can affect assay performance, possibility resulting in an underestimation of shedding or a false negative result [24]. For the detection of infectious virus, the FDA recommend assays that measure infectivity in terms of Tissue Culture Infectious Dose 50 ($TCID_{50}$), plaque-forming units (PFU) or focus-forming units (FFU) [23].

In the AAV-specific context, if the presence of replication-competent recombinants or intact virus particles are suspected in shed material an infectivity assay should be developed. These assays can be problematic as AAVs do not induce a cytopathic effect in cell culture and may not be infectious despite internalization [32]. A suggested approach is to treat a susceptible cell line with shed material in the presence or absence of helper virus followed by assessment of viral internalization by qPCR [32].

Storage conditions for samples obtained for shedding and biodistribution analysis also need to be taken into consideration, especially given that requirements may vary due to differences in product stability in different matrices. Multiple aliquots may also be required for different tests (e.g., qPCR for nucleic acid detection and infectivity assays) [23]. FDA guidance suggests that degradation of viral or bacterial nucleic acids in enzyme-rich clinical samples can be accounted for by spiking of mock/donor samples with a reference standard shortly after collection in order to determine the percentage recovery – such samples should be collected, stored, shipped, and extracted in the same way as the 'test' samples.

APPROACHES FOR A HARMONIZED APPROACH FOR THE ASSESSMENT & REPORTING OF BIODISTRIBUTION & SHEDDING

Since 2008, the US National Institutes for Health have supported the National Gene Vector Biorepository (www.NGVBCC.org) as a means to collect data from pharmacology/toxicology studies, archive reagents and samples from nonclinical studies, and in some cases, offer core analytical tests typically employed in nonclinical and clinical studies for gene therapies [37]. The organization has distributed over 1,000 reagents and collected over 36,000 specimens from nonclinical viral gene therapy studies. Furthermore, it maintains a searchable database of gene therapy pharmacology/toxicology studies ultimately resulting in 114 publications since 2018. As of 2020, the pharmacology/toxicology database contained information from 52

TABLE 4

Summary of expectations for shedding in the context of environmental risk assessment (EMA)/environmental assessment (US).

	EU	US
Legislation	**Pharmaceutical legislation** ▶ Directive 2001/83/EC (as amended by Directive 2009/120/EC) **Environmental legislation** ▶ Directive 2001/18/EC "Deliberate Release" ▶ Directive 2009/41/EC "Controlled Release" ▶ Directive 2000/54/EC - protection of workers from risks related to exposure to biological agents at work	The FDA must comply with the National Environment Policy Act of 1969 when considering both IND and BLAs
Relevant guidance document(s)	▶ Environmental Risk Assessment for Medicinal Products containing, or consisting of, Genetically Modified Organisms (GMOs) (Module 1.6.2) [26] ▶ Good Practice on the assessment of GMO related aspects in the context of clinical trials with AAV clinical vectors [27]	FDA Guidance for Industry - Determining the Need for and Content of Environmental Assessments for Gene Therapies, Vectored Vaccines, and Related Recombinant Viral or Microbial Products [29]
CTA / IND expectations	▶ In accordance with the environmental legislation, an environmental assessment is required for GMOs such as investigational GTMPs to ensure compliance with GMO legislation (either "Deliberate Release" or "Contained Release" Directives, depending on Member State). ▶ Authorization is granted on the basis of the submitted environmental assessment ("GMO application") by the relevant environmental authority before a clinical trial can begin in each Member State[1] ▶ Good Practice documents and Common Application Forms, adapted to the specific characteristics of AAVs are available, and have been endorsed by a majority of Member States. In the context of AAVs, specific requirements with regard to shedding data are not detailed. However, potential environmental hazards, including to human and animal health, are identified, which could arise from unintended transmission of clinical vectors ▶ The common application form for AAVs contains a section where detailed data on clinical vector shedding should be included to ensure an appropriate evaluation of environmental risk ▶ In the context of GMO applications for AAVs, if there is no prior clinical experience with the vector, a discussion of the potential for shedding based on nonclinical data should be provided according to the common application form for investigational medicinal products for human use that contain or consist of AAV vectors [28]. In addition, clinical experience from related clinical vectors can be included to support the environmental risk assessment – the relevance should be justified based on dose and route of administration in particular.	A "categorical exclusion" can be claimed for IND submissions for GTMPs, based on the assumption that any potential effect on the quality of the environment would not be significant due to close monitoring and a limited number of treated patients. Therefore, no environmental assessment is generally required to support an IND submission.
MAA / BLA expectations	▶ An Environmental Risk Assessment (ERA) must be included in a MAA for all medicinal products and the legislation (Directive 2001/83/EC, as amended by Directive 2009/120/EC) contains specific requirements for information on the shedding of GTMPs due to their potential for transmissibility / infectivity. ▶ The ERA should include an assessment of the potential for transmission to third persons and clinical studies should include an analysis of shedding to address product excretion. ▶ In addition to a potential adverse effect on human health through transmission to third persons, shedding is also considered a factor that could produce an adverse effect on the environment through transfer to other species or possible interference with other prophylactic or therapeutic medical treatments (e.g., transfer of antibiotic resistance genes), and data on shedding is therefore required to appropriately evaluate environmental risk and mitigate it if necessary [26]. ▶ ERA submitted with a MAA is evaluated in consultation with the national bodies responsible for the release of GMOs into the environment	▶ For BLAs, any GTMP which is not considered to occur naturally in the environment (i.e., any GTMP expressing one or more protein coding sequences from a genus different from the organism expressing the sequence) should include an Environmental Assessment (EA) in the BLA. ▶ FDA recommends that an analysis of shedding demonstrating the release of vector DNA and / or infectious virus should be included in the EA – based on these data the potential effects on the environment are then considered. For example, if no infectious virus is detected in shed material, a justification can be made that only the environmental effects of vector DNA should be considered. ▶ The shedding of potential variants, which may pose greater environmental risk, should also be considered in the EA (e.g., in the context of AAV, the presence of replication-competent virus due to recombination events during the manufacturing process).

Note: 1 For further information and discussion regarding the variability in the timelines and application of the GMO legislation between EU Member States, which can result in delays in clinical development, particularly in multi-centre studies, please refer to a recent review by EFPIA [2].
AAV: Adenoassociated Virus; BLA: Biologics License Application; CTA: Clinical Trial Application; EA: Environmental Assessment; EU: European Union; FDA: Food and Drug Administration (US); GMO: Genetically Modified Organism; IND: Investigational New Drug; MAA: Marketing Authorization Application.

anonymized nonclinical studies and is intended to foster data sharing between sponsors in the interest of comparing data, such as biodistribution, of similar viral serotypes and routes of administration. While this has been an outstanding effort to collect a broad array of information from gene therapy sponsors, we propose to take a deeper dive into the details of nonclinical biodistribution and shedding studies, further collating study design aspects as well as analytical output. Such outputs would enable comparison between studies and could permit extrapolation of existing data for use in regulatory submissions of similar class vectors, thus reducing animal usage and ultimately accelerating gene therapies into the clinic.

In November 2020, the Accelerating Research & Development for Advanced Therapies (ARDAT) consortium began. It represents a collaboration between academia, small and medium-size enterprises (SMEs) and the European Federation of Pharmaceutical Industries and Association (EFPIA) members funded by the European public/private partnership Innovative Medicines Initiative (IMI) and EFPIA 'in kind' contributions. The project involves 12 industry members, 7 SMEs and 16 academic institutions to conduct five years of intensive research into AAV biology in the hopes that lessons learned will facilitate increased and more effective AAV therapies in the clinic. The project is organized in five 'work Packages', each with a particular focus including immune responses to AAVs, the metabolism of the AAV genome after cellular transduction and the development of a biobank of human samples from both AAV and non-AAV clinical trials, which will feed into these research questions. The final work package will focus on engagement with regulatory authorities to ensure that the data generated by the project will support recommendations for regulatory harmonization and create predictable regulatory pathways for innovation.

One initiative within the regulatory work package is to gather information on biodistribution and shedding in the public domain with the intent of collating data from various studies in a database. By formally organising biodistribution and shedding data in this way, developers utilizing the same viral serotype, route of administration, dose levels, etc., could leverage this data to accelerate product development. It is hoped that this leveraged data may be sufficient to satisfy regulatory expectations for the description

TABLE 5

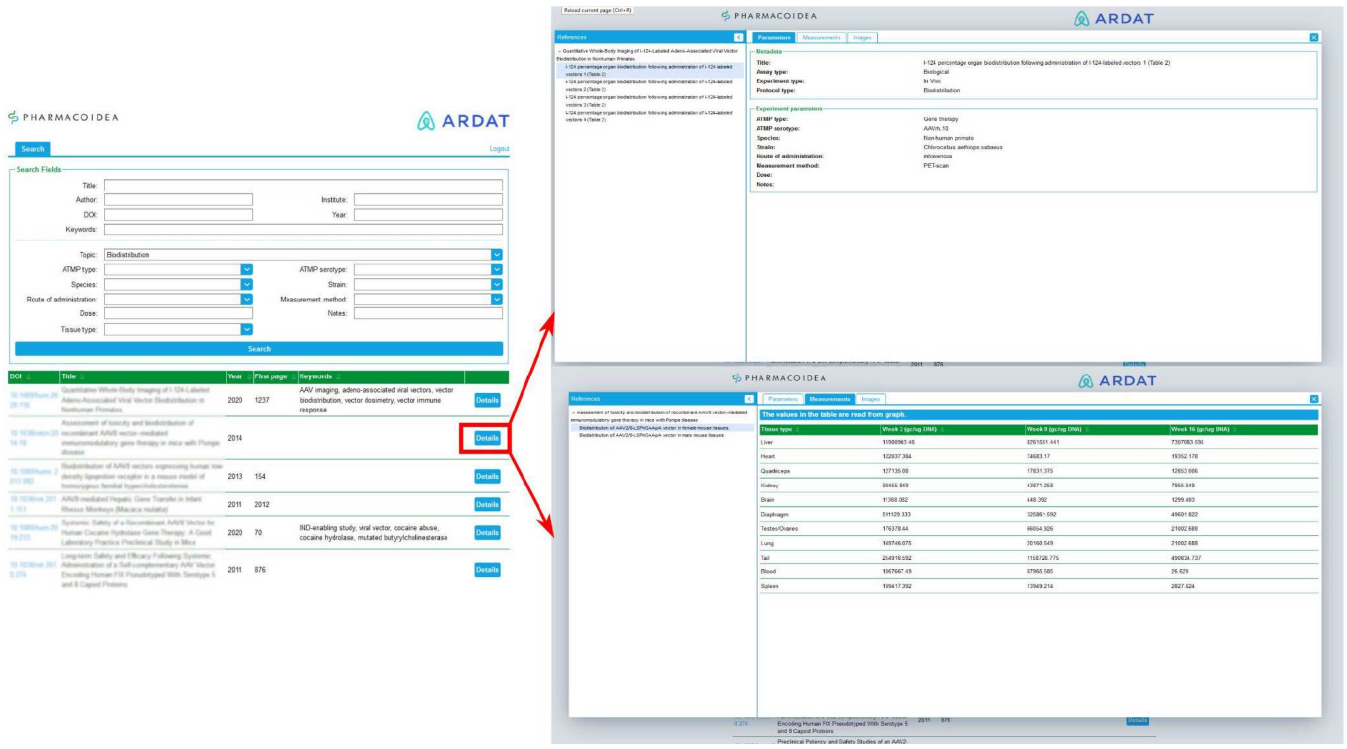
Application of shedding requirements to AAV vectors.

Biological property	Consequences for shedding	Considerations for AAV
Replication competence	Replication-competent vector may persist in the patient for extended periods and may increase in amount over time. Shedding potential of replication-competent viruses can be higher, resulting in a higher probability of transmission	Wild-type AAVs are naturally replication deficient and require co-infection with helper viruses to replicate. Furthermore, recombinant AAVs also lack the rep and cap genes, which are typically replaced by the therapeutic transgene. Consequently, rAAV-based vector shedding is predicted to be of short duration. Replication-competent recombinants potentially produced during rAAV manufacturing may need to be considered.
Duration of infection / vector persistence	Short-term infection and/or rapid viral clearance due to immune response may reduce the duration and extent of vector shedding	Long-term infection and therefore shedding may occur in patients receiving immunosuppressive regimens, a second peak of shedding may occur if the regimen is discontinued.
Properties of parental viral vector (e.g., route of transmission)	There could be an increased risk of transmission in excreta or secreta corresponding to the natural route of transmission	For AAVs, there is potentially more risk of transmission from nasopharyngeal secretions rather than urine or faeces

AAV: Adenoassociated virus; rAAV: Recombinant adenoassociated virus.

FIGURE 1

Example of biodistribution database functionality.



In the left panel headed "Search Page" the user has input search parameters to obtain literature articles which contain data regarding AAV9 biodistribution in non-human primates. After clicking on the "Details" button (indicated by the red square), information regarding a specific article can be obtained, including detailed experimental parameters and experimental data.

TABLE 6

Summary of shedding data submitted to support EMA marketing authorization of AAV-based GTMPs.

Product/Reference documents	Vector subtype (transgene)	Therapeutic indication	Posology/Route of administration	Shedding data to support marketing authorization
Luxturna (Voretigene neparvovec) [17]	AAV2 (hRPE2)	Treatment of adult and paediatric patients with vision loss due to inherited retinal dystrophy caused by confirmed biallelic RPE65 mutations and who have sufficient viable retinal cells.	1.5×10^{11} vg/eye Subretinal injection	**Nonclinical** ▶ Viral shedding was not described in animals. **Clinical** ▶ In Phase I and Phase III clinical studies, shedding of AAV2-hRPEv2 in tears was reported in approximately 55% (17/31) of treated patients. Shedding was transient in nature, with the majority of positive samples were seen between one and three-days post-administration. ▶ Low levels of vector were also detected in serum of some patients, up to 14 days post-administration.
Zolgensma (onasemnogene abeparvovec) [20]	scAAV9 (SMN1 gene)	Treatment of patients with 5q Spinal Muscular Atrophy (SMA) with a bi allelic mutation in the SMN1 gene and a clinical diagnosis of SMA Type 1 or patients with 5q SMA with a bi allelic mutation in the SMN1 gene and up to 3 copies of the SMN2 gene	1.1×10^{14} vg/kg Intravenous infusion	**Nonclinical** ▶ No nonclinical shedding data was included in the dossier, data on shedding in humans based on the published literature was presented, which was considered sufficient. **Clinical** ▶ Data from clinical studies showed that the vector was primarily cleared from the body in stool for up to 60 days post-administration. Low levels of shedding in urine and saliva were reported at 1-day post-dosing. **Environmental risk assessment** ▶ The risk associated with shedding of viral particles and potential third-party transmission was considered to be low; the SMN1 transgene was not considered immunogenic or toxic, and AAV infection would be asymptomatic. ▶ The worst-case scenario was considered to be the spread of replication-competent AAV expressing SMN1 arising from recombination during manufacturing or co-infection with wtAAV in the patient's cells. However, negligible risk was assigned to this scenario due to the lack of evidence for a direct effect of SMN1 on viral biology and pathogenicity, and the limited capacity of AAVs which precludes packaging of rep, cap and SMN1 genes in the same vector.
Glybera (alipogene tiparvovec)[1] [21]	AAV1 expressing human lipoprotein lipase	Familial lipoprotein lipase deficiency (LPLD)	Intramuscular injections in the legs, 1.5×10^{12} vg per injection site	**Nonclinical** ▶ Shedding was not addressed in nonclinical studies **Clinical** ▶ Shedding was assessed in the clinical studies by collecting saliva, urine and semen. In one clinical study faeces was also collected. After administration of Glybera to the participants, the highest vector DNA concentrations were detected in the serum, with clearance by one to two logs per week. In saliva vector DNA was still detectable up to 12 weeks; in urine up to 10 weeks and in semen up to 26 weeks. All but two patients received immunosuppressants for 12 weeks. There is the theoretical risk that the co-administration of the immunosuppressant regime leads to longer persistence of virus DNA in serum and as well to longer shedding in saliva, urine and semen. High levels of vector DNA were observed up to 12 months after dosing in the target tissue for Glybera, injected leg muscle, but not in non-injected muscle.

Note: 1 The marketing authorization for Glybera expired in 2017, following the decision of the marketing authorization holder not to apply for a renewal due to a lack of demand for the product.
AAV1: Adeno-associated virus type 1.

and communication of biodistribution and shedding data to health authorities, or at least drive the design of more focused studies that could be reduced in size, scope and length.

Methodological aspects for the construction of a database to collate biodistribution & shedding data

While more recently new AAV viruses with either novel or ancestral capsid proteins have shown enhanced targeting and high selectivity for key tissues [38], for many years the core set of AAV capsids – 2, 5, 8 and 9 – were used in a variety of research and development programs. In addition, the routes of administration, dose levels and species have generally remained within a small range of choices. It has already been suggested that the field of AAV gene therapy could take advantage of this plethora of information and leverage published data for a particular serotype, route of administration, dose level and species to permit the reduction or elimination of further nonclinical biodistribution assessments, thus minimising animal usage and streamlining nonclinical development programs [4].

We describe here the initial stages of the construction of a database of published biodistribution and shedding data for AAVs. By collecting a core set of metadata relating to how the study was performed as well as the experimental data, it is hoped that the database will formalize the currently existing data on biodistribution and shedding of AAVs.

An example of biodistribution database functionality is shown in Figure 1. Such information could be made publicly available to developers of AAV-based therapies where data could be leveraged in regulatory applications, and depending on the depth of information available, potentially permit dedicated biodistribution analysis for particular products to be waived. As the field evolves and understanding of AAV biology progresses, we anticipate that newly generated data for the aforementioned 'core' serotypes will be complemented by data on new serotypes or routes of administration as the database grows.

To initially populate the database, a search was conducted on the PubMed database (https://pubmed.ncbi.nlm.nih.gov) using the search terms 'AAV' AND 'biodistribution', which yielded 122 potentially relevant abstracts. A complimentary second search with the terms 'AAV gene therapy' AND 'biodistribution' was conducted and yielded 107 potentially relevant abstracts. After screening of each abstract for relevance by members of the ARDAT consortium, a total of 102 relevant articles were identified. These articles were uploaded into a shared reference manager (https://sciwheel.com) where consortium members conducted a detailed review of each article and identified the data to be uploaded to the database.

Our first priority was to identify and collect quantitative data on vector distribution (e.g., genome copy (gc) amount, gc/µg DNA or gc/mg tissue), which on most occasions was found within tables and figures. After consultation with ATMP experts within the ARDAT consortium, a series of fields for data entry were designed to capture all relevant information with regard to study parameters. The main general parameters include ATMP class, ATMP serotype, Species, Strain, Route of Administration. Parameters specific for biodistribution and shedding include Measurement method and Tissue type. These parameters appear in the browser interface as dropdown lists to filter search results with the possibility to select any number of parameters. To recover exact numerical data from plots and images, we used a web-based application (https://apps.automeris.io/wpd/). The topics and their parameters are flexible, such that database can be expanded, for example with immunogenicity information in the future.

Need for data standards & minimum requirements for reporting & publishing studies

During construction of the database, we identified inconsistencies across the literature which may limit the usefulness of extrapolation of these data to support regulatory submissions of similar class vectors.

Areas of inconsistency include, for example, the units of measurement used, e.g., results presented in vector genome/µg (vg/µg, or any other mass) and results given in vg/diploid genome or vg/*x* amount of DNA make comparisons difficult across the literature. There are many less frequently used units (vg/cell, vg/eye) employed without referencing mass of DNA used to calculate cell number. Similarly, the AAV terminology is reported in various ways, for example, an AAV 2 rep gene with AAV 5 cap gene is reported as AAV2/5 [39], AAV5 [40] and AAV2.5 [41], which causes confusion.

Another major inconsistency is in the type of tissue analysed and the timepoints and which measurements are taken. For example, measurement of biodistribution can vary between whole tissue or many parts of a single tissue type [42–44]. A standard set of tissues for biodistribution measurements would allow these data to be referenced in regulatory submissions and prevent duplicative nonclinical work. The most recently recommended tissue list from health authorities is outlined in the IPRP reflection paper on conduct of biodistribution studies [16]. In addition, standard tissues expected to be collected are also outlined in the current draft version of ICH S12 [3]. It is unlikely that there will be further granular guidance from health authorities on topics such as how tissues are collected, fractionated and stored.

We noted that data from infectivity studies are rarely reported, and where it is reported there is not always a description of methods.

Finally, our work revealed discrepancies in the reporting of the bioanalytical methods used, including a general lack of reported validation parameters e.g., limit of detection. There were also varied approaches to the reporting of sampling profiles and schedules. Such variations in reporting render the experiments difficult to interpret and reproduce.

In an attempt to illustrate this point, we conducted an exercise from the point of view of a hypothetical developer of an AAV9-based gene therapy intended for intravenous administration in the clinical setting. Using the search filters 'AAV9' for ATMP serotype and 'intravenous' for route of administration, we identified 12 articles currently in the database, which reported biodistribution data derived from qPCR analysis for AAV9-based vectors after intravenous administration.

The majority of published studies (9 of 12; 75%) were conducted in mice (either wild-type strains, namely Balb/c or C57BL/6 [7 studies] or specific disease models [one study each in Barth syndrome and dystrophin-deficient mouse models]). The remainder of the studies were conducted in NHPs (3 of 12; 25%). With regard to pre-existing immunity, none of the studies in mice included an analysis of this issue, whilst 2 of the 3 studies in NHPs included such an analysis. Only one study in NHPs included animals with and without pre-existing immunity [45].

It was noteworthy that none of the studies included information with regard to bioanalytical method validation, including the effects of different tissue matrices, nor were limits of detection stated. In general, biodistribution of vector genomes was only analysed at one timepoint, which showed a wide variation; ranging from 10 days to 5 months in mouse studies and 28 days to 2 years in NHP studies. Such wide ranges could be explained by the desire to limit animal use and study costs and the use of complementary approaches to examine transgene expression as described in the next paragraph.

A significant proportion of studies used a reporter transgene to facilitate the analysis of transgene expression over several timepoints (4 of 12 studies; 33%). Detection methods included *in vivo* imaging of live animals using luciferase [46] or analysis of secreted enzyme activity [45]. These analyses of transgene

expression complemented the vector genome biodistribution data obtained from tissues harvested at the terminal timepoint. The use of GFP transgenes also facilitated analysis of transgene expression in specific cell types / areas of tissue, for example in the study of Weber-Adrian and colleagues [47].

Vector genomes were detected using primer sets to detect either sequences in the parental vector (3 of 12 studies; 25%), transgene (7 of 12 studies; 58%) or were not stated (2 of 12 studies; 17%). It is notable that none of the studies appeared to use primer sets at junctional regions that would permit detection of both parental vector and transgene sequences.

There was relatively little consistency with regard to the units of quantification used to expressed vector genome biodistribution. Viral genome copies / vector copies / transgene copies were expressed per µg of genomic DNA / per diploid genome / per cell or per copies of reference gene. There was also a wide variation of reference genes used to quantify genomic DNA (e.g., where it was stated in the article, the following genes were used as references for genomic DNA quantification; NCAM, beta-actin, lamin B2, titin, epsilon-globin).

More consistency was seen with the panels of tissues analysed, vector genome distribution to heart was reported in all studies and liver in 11 of 12 studies (91%). Other tissues commonly analysed included lung (10 of 12 studies; 83%), kidney (7 of 12 studies; 58%), and brain (7 of 12 studies; 58%).

Once the database is constructed, the next step of this initiative will be to publish recommendations in analytical and reporting standards, such that data sharing across groups will be more universally interpretable.

CONCLUSION

Recommendations for biodistribution and shedding studies have been issued by regulatory authorities across the globe, which has resulted in points of divergence and complexities in the global development of gene therapies. Whilst the 2021 release of the draft ICH Guideline S12 is a welcome step toward harmonization, further opportunities exist. Extensive nonclinical work over the course of the last three decades has generated a plethora of literature on the nonclinical biodistribution and shedding of AAV vectors, such that regulatory applications should rely on this data set and reduce animal usage. Inconsistencies in reporting standards across the field has hampered the usefulness of extrapolation of published work to support regulatory submissions of similar class vectors. A publicly available database of biodistribution and shedding data, with established minimum data standards for reporting and publishing data will greatly facilitate regulatory convergence and nonclinical development by avoiding repetitive studies and reducing animal usage which ultimately will accelerate these important therapies to market.

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