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RESEARCH ARTICLE

Regulatory Trends in the Nonclinical Development of Viral Vector-Based Gene Therapies: A Benchmark Analysis of Approved Products

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ABSTRACT

As it is often the case with innovative technologies, regulatory agencies are highly demanding in product safety demonstration from those pioneering breakthrough therapy products. Since the first historically approved gene therapy medicinal product (Gencidine in 2003) by the Chinese National Medicinal Products Administration, gene therapy medicinal products have slowly been emerging in other regions, as illustrated by the first European-approved gene therapy medicinal product (Glybera in 2012) and the first US-approved product (Imlygic in 2015). From then, with the rise of new molecular technologies (*e.g.,* non-viral and viral vector systems), an exponential growth of gene therapies development could be gauged with, for example, the approval of more than thirty gene therapies between 2016-2022.

Using a method based on Preferred Reporting Items for Systematic reviews and Meta-Analyses principle, throughout different literature databases, this review is restricted to the evaluation of viral vector-based gene therapy medicinal products (VV-GTMPs). It also considered relevant guidelines and public assessment reports issued by the EMA and / or the FDA on the products these agencies approved. Then, a benchmark was performed to help stakeholders to identify regulatory trends and to design appropriate nonclinical programs establishing the benefit / risk ratio for patients to be enrolled in clinical trials. The analysis was focused on the nonclinical activities (pharmacology, biodistribution / persistence / shedding, and toxicology) performed by Applicants / Sponsors.

As of 30 March 2023, 18 VV-GTMPs have been authorized by the EMA and 14 by the FDA to treat either orphan diseases or limited number of oncology patients. The majority of these therapies are based on adeno-associated or retroviral viruses (often lentiviruses able to transfect hematopoietic $CD34^+$ cells or T-cells). Based on an analysis of the ongoing clinical trials, there is now a trend for developing gene therapies for larger patient populations.

In conclusion, given the VV-GTMPs diversity and targeted indications, a "one-size fits all" nonclinical development plan cannot be considered by default. Instead, individual, risk-based, tailored nonclinical development programs appear more appropriate to assess such products, taking into consideration the lessons learned from the past. In such fast-evolving environment, regulatory agencies need to adapt their evaluation process very rapidly.

INTRODUCTION

There is a worldwide consensus on defining gene therapies as an intervention in which a defective gene is repaired or replaced either *in vivo (in situ)* by direct administration of a genetic construct using a clinically safe vector into patients, or *ex vivo* by modifying cells before their administration to the patients. Several health regulatory agencies indeed propose comparable definitions:

- The European Medicines Agency (EMA) defines a gene therapy medicinal product (GTMP) a product consisting of "*a vector or delivery formulation / system containing a genetic construct engineered to express a specific transgene (therapeutic sequence) for the regulation, repair, replacement, addition, or deletion of a genetic sequence. By using gene therapy constructs in vivo, genetic regulation or genetic modification of somatic cells can be achieved in situ. Gene therapy vectors can be used ex vivo for the manufacture of genetically modified cells*"[1].
- The US Food and Drug Administration (FDA) defines gene therapy products as products "*that mediate their effect by transcription and/or translation of transferred genetic material and / or by integrating into the host genome and that are administered as nucleic acids, viruses, or genetically engineered micro-organisms. The products may be used to modify cells in vivo or transferred to cells ex vivo prior administration to the recipient*"[2].
- The Pharmaceuticals and Medical Devices Agency (PMDA) in Japan defines gene therapy products as products comprising "*in vivo gene therapy products and ex vivo genetically modified human cell therapy products (ex vivo gene therapy products) among regenerative medical products.*"[3].
- In Australia, Therapeutic Goods Administration (TGA) uses the following definition for gene therapies: "*substance used in or administered to human beings to regulate, repair, replace, add or delete a genetic sequence and any substance involved in the therapeutic, prophylactic or diagnostic effect of the product*"[4].
- For Chinese National Medical Products Administration (NMPA, former Chinese FDA), gene therapies are classified based on the gene modification approach, either *ex vivo* or *in vivo*[5].

As the effects of gene therapy are expected to be long-lasting, the need of repeated interventions is not expected. Nevertheless, the development procedures, aiming at restoring the function of a defective gene or replacing a mutant gene, remain challenging, as the safety evaluation of such therapeutic solutions. Currently, most of gene therapy products in development (74%)[6] concentrate on only few medical specialties (oncology, hematology, endocrinology, neurosciences, cardiology, and ophthalmology). Those gene therapies cover eight disease families (inborn errors of metabolism, coagulation defects or related conditions, primary disorders of muscles, disorders of retina, anemias or other erythrocyte disorders, movement disorders, diseases of arteries or arterioles, and primary immunodeficiencies)[6].

Focusing on approved viral vector-based GTMPs (VV-GTMPs) in the EU and/or US (benchmark-approach), the aim of this literature review is to help stakeholders to identify the regulatory trends in the field and design appropriate nonclinical programs to demonstrate the expected positive benefit / risk ratio for patients and insure the future Marketing Authorization Application (EU) and / or Biological License Application (US) processes.

METHOD

Approach

An electronic search of authorized viral vector-based gene therapy medicinal products (VV-GTMPs) from EMA and FDA databases was conducted at first. As far as applicable, the selection of relevant documents was then performed using a method based on Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) principle[7], in conducting and reporting a systemic review. Scientific literature searches for authorization, discontinuations, withdrawing, approvals using EMA and FDA databases, MEDLINE / PubMed, Cochrane Library and EMBASE databases were also conducted. In addition to regulatory publications, additional articles search was done using keywords such as gene therapy, gene therapies, viral-vector, marketing authorization, regulatory and approval / approved. All searches were conducted until 30 March 2023.

Selection of publications and regulatory documents.

The stepwise approach screening of the documents is illustrated in **Figure 1**.

First, the search conducted with the EMA and FDA databases identified a total of eighteen (18) gene therapies approved in Europe, fourteen (14) were approved in the US. During this first analysis,

products approved in other regions were also identified, such as 5 were approved in Japan, and 4 in Australia[8] and some in other countries such as Philippines and South-Korea (**Table 1**, **Table 2** and **Figure 2**).

A broad reference screening using EMBASE database led to the identification of 140,290 articles referring to gene therapy. After excluding articles that did not mention viral vectors, the remaining references corresponded to a number of 9,939 articles. The final filter aimed to exclude the articles that did not mention any of the approved VV-GTMPS identified from EMA and FDA databases, using their brand names (**Table 2** and **Figure 1**). After this final screening, 82 references were kept for further analysis.

A third analysis using EMBASE aimed to identify references that shared the terms 'gene therapy', 'market authorization' and 'regulatory approval'. After exclusion of articles not responding to the selection criteria, a total of 27 articles were identified (**Figure 1**). After verification, these 27 references were already included in the 82 references identified during the previous step.

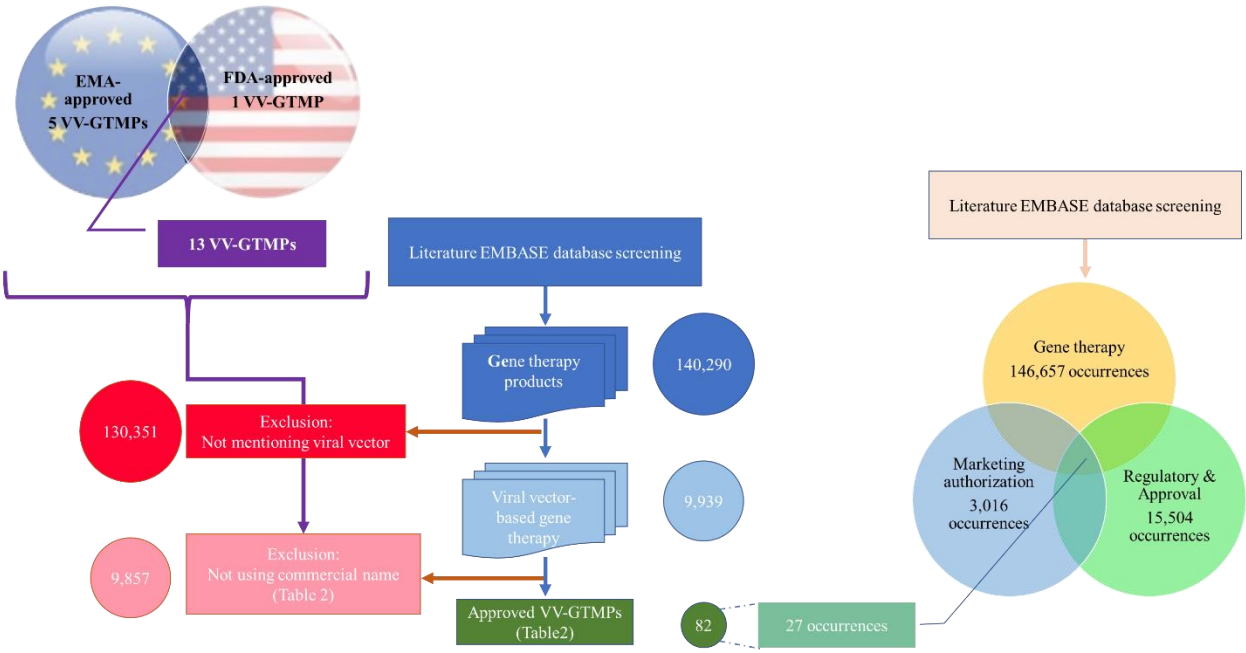

Figure 1. Flowchart summarizing the multistep literature search.

RESULTS

The current situation

Over a period of two decades, and despite the efforts to launch new products, only few gene therapies have been approved by health authorities worldwide. From those products, targeting monogenic human disorders and different forms of cancers, some are based on viral systems delivering therapeutic genes to target tissues, and other are based on nonviral delivery systems (**Table 1**)[9].

Viral vectors played a leading role in gene therapy because of their superior gene delivery capacity compared to non-viral vectors[10].

Table 1. Approved Gene Therapies as of 30 March 2023

Systems	Technology	Products
Viral	Adenoviral-based gene delivery	Gencidine, Oncorine,
	Therapeutic AAVs	Glybera*, Luxturna, Zolgensma, Roctavian
	HSV & retroviral delivery systems	Imlygic, Rexin-G
	LVV delivery system	Zynteglo*, Kymriah, Breyanzi, Libmeldy, Abecma, Skysona*
	Retroviral based products	Strimvelis, Zalmoxis*, Yescarta, Tecartus
Non-viral (not discussed further)	Naked DNA	Neovasculgen, Collatgene
	Therapeutic oligos based on RNAi technology	Onpattro, Givlaari
	Therapeutic oligos modulating splicing events	Exondy51, Spinraza, Amondys 45, Vyondis 53; Viltepso, Defitelio
	Antisense oligos (ASO) with RNAse H activity	Vitravene, Kynamro, Waylivra, Tegsedi
	Aptamers modulating protein activity	Macugen, Defitelio

Notes: *withdrawn by marketing authorization holder for commercial reasons[11-14].

The first historically approved VV-GTMP, Gencidine, was approved by the National Medicinal Products Administration (NMPA, former Chinese FDA) in 2003 (**Table 1** and **Figure 2**). Gendicine was a recombinant human p53 adenovirus, developed by Shenzhen SiBiono GeneTech Co. Ltd., as a first-in-class gene therapy product to treat head and neck cancer, and entered the commercial market in 2004[15].

Another example of early approval is illustrated by Rexin-G® which was granted an accelerated approval for the treatment of all chemotherapy-resistant solid malignancies in the Republic of the Philippines in 2007 (**Table 1** and **Figure 2**).

Since then, VV-GTMPs have slowly been emerging in other regions, as illustrated by the first EU-approved VV-GTMP (Glybera in 2012, **Table 3-1**) and the first US-approved product (Imlygic in 2015, **Table 4**). With the development and adoption of new viral vector systems, an exponential growth could be gauged with, for example, the approval of more than thirty VV-GTMPs between 2016-2022.

In 2012, the first gene therapy product Glybera, based on an AAV delivery approach, was approved in EU for patients suffering from lipoprotein lipase deficiency but was withdrawn in 2017 as per request of the MAA holder, Amsterdam Molecular Therapeutics (lack of commercial viability)[6,13]. Similarly, Bluebird bio announced in 2021 that they were giving up the commercialization of Zynteglo (**Table 6**) in the EU, because European payors did not accept to pay the $1.8 million price-tag to threat beta-thalassemia[12].

In 2016, Strimvelis, a retrovirus-mediated gene therapy product approved in Europe, was used to treat patients suffering from severe combined immune deficiency (**Table 5**)[16].

In 2017 Kymriah (tisagenlecleucel, lentivirus mediated, **Table 7-1**) and Yescarta (axicabtagene ciloleucel, based on a retroviral vector, **Table 7-1**) were approved by US FDA[17]. Both products were also approved later in Europe in 2018[18,19].

The first FDA-approved AAV based (AAV2) product was Luxturna (voretigene neparvovec, **Table 3-1**)[20] for the treatment of inherited retinal dystrophy. It was also approved in Europe in 2018[21]. In 2019, the AAV-based (AAV9) gene therapy Zolgensma (onasemnogene abeparvovec, **Table 3-1**) was approved by US FDA[22] to treat patients suffering from spinal muscular atrophy (SMA). Product approval occurred in 2020 in Europe[23]. As of March 2021, Zolgensma has been approved in 38 countries dans more than 1,000 patients were treated both across clinical trials and commercial settings.

Among the approved VV-GTMPS, adeno-associated virus (AAV)-based transgene delivery remains the most common *in vivo* delivery system, while *ex vivo* delivery predominantly relies on transduction of hematopoietic stem cells with lentiviral vectors (LVs). The increased use of AAV in gene therapy is based on the ability of AAV vectors to reach target tissues. The ongoing discovery and study of various AAV serotypes increasing tissue or cell specificity and having less immunogenicity, offers huge possibilities. However, AAV only have small packaging capacity[24], and will not become the sole vectors used for gene therapy in the future. *Ex vivo* delivery (using mainly retrovirus, 61%, or lentivirus, 29%) is the technology of choice when gene therapy carriers are cells of hematopoietic

lineage, this predominance of retroviral vectors (RV) / LV reflects that those vectors represent the right tool for a large subset of targeted cell types (*i.e.*, T cells and hematopoietic stem cells, **Table 5** and **Table 6**).

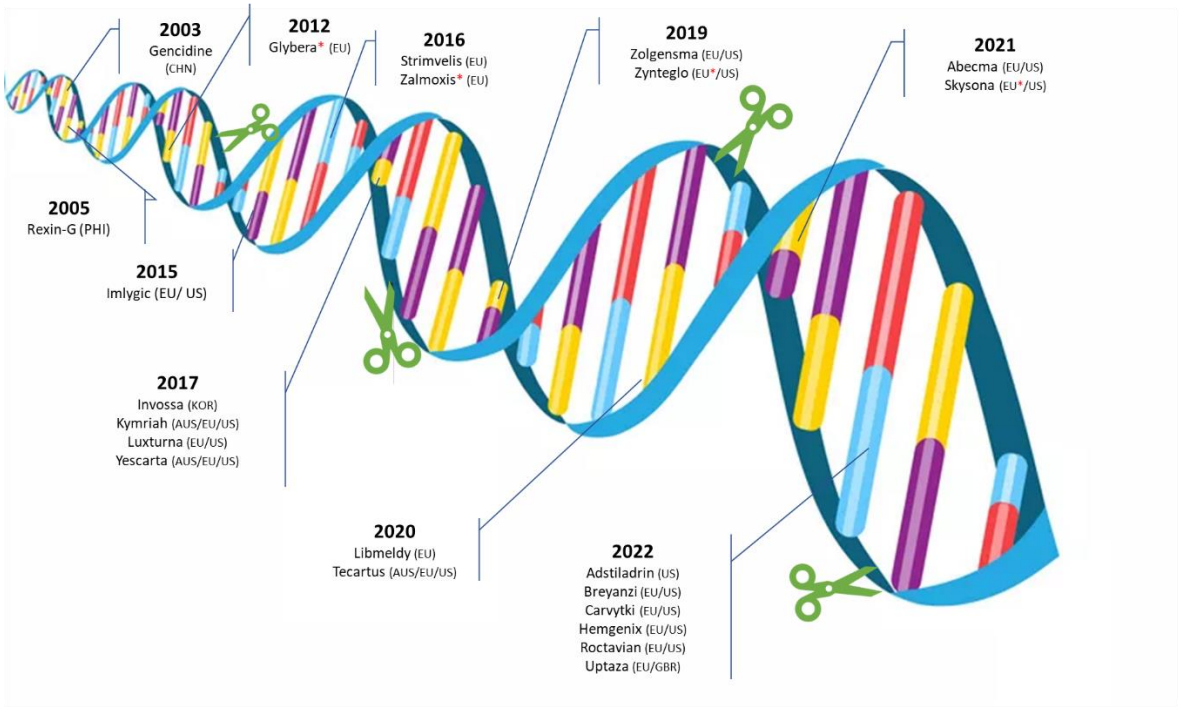

Figure 2. Viral vector-based Gene therapy: Chronological worldwide approvals
Notes: The approved VV-GTMPs are indicated by their commercial names, with the approval regions indicated in brackets. The red asterisk (*) indicates product that have been withdrawn and are no longer authorized.
Approval region acronyms: AUS: Australia (Therapeutic Goods Administration, TGA); CHN: China (National Medicinal Products Administration, NMPA formerly China FDA); EU: Europe (European Medicines Agency, EMA); GBR: United Kingdom (Medicines and Healthcare products Regulatory Agency, MHRA); KOR: South Korea (Ministry of Food and Drug Safety, MFDS); US: United States (US Food and Drug Administration, FDA).

Table 2. Approved VV-GTMPs in EU and US

EMA-Approved	EMA- & FDA-Approved	FDA-Approved
Glybera*	Abecma	Adstiladrin
Roctavian	Breyanzi	
Strimvelis	Carvykti	
Upstaza	Hemgenic	
Zalmoxis*	Imlygic	
	Kymriah	
	Libmeldy	
	Luxterna	
	Skysona*	
	Tecartus	
	Yescarta	
	Zolgensma	
	Zynteglo*	

*:Withdrawn in EU, i.e. Skysona,[11] and Zynteglo[12], Glybera[13], Zalmoxis[14].

The vast majority (93%) of *ex vivo* gene therapies only target a single gene.
Of note, only nonclinical activities conducted for approved viral vector-based GTMPs (VV-GTMPs) will be further discussed in this review. In addition, a special focus will be done on products approved in the EU and the US.

As of 30 March 2023, the screening from EMA and FDA databases led to identifying 18 VV-GTMPs have been authorized by the EMA[25] and 14 by the FDA[26]; some products being approved by both (see **Table 2** and **Table 3** to **Table 7**, **Figure 2**).

Principles for nonclinical evaluation

Considering the list of approved VV-GTMPs and the associated regulatory guidelines and documents issued upon their authorization in the EU (European Public Assessment Reports, EPARs) and US (Summary Basis for Approval, SBA), the authors of this retrospective review analyzed the nonclinical activities (*i.e.,* pharmacology, biodistribution / persistence / shedding, and toxicology) performed by Applicants/Sponsors for the approval of these VV-GTMPs.

The aim of the nonclinical program, along gene-therapy medicinal product development, is to provide sufficient relevant information to properly assess the benefit / risk for patients[27].

The nature of the nonclinical development is directly linked to the nature of the VV-GTMP product. It will also depend on the clinical intended use, targeted clinical population, route of administration and dose regimen, and ultimately on the availability of relevant nonclinical models. Nonclinical studies could be carried out either as stand-alone or combined studies. In the case VV-GTMPs, a particular attention should be paid to select the suitable control groups within the nonclinical studies, based on knowledge about the vector used.

All studies should be designed with the most pharmacologically appropriate model (*in vitro, ex vivo,* or *in vivo*), to allow intended vector transfection / transduction / infection and replication. Evidence on the potential clinical effect or on the related biological effect or molecular mode-of-action of the VV-GTMPs should be demonstrated. Primary pharmacodynamic studies should inform on how the nucleic acid sequence reaches the intended target (organ or cells) and the intended function (level of expression and functional activity).

Safety pharmacology studies aim to investigate the potential undesirable effects of the vector on vital physiological functions (respiratory, cardiovascular, and central nervous systems). Therefore, appropriate safety pharmacology studies, as per ICH S7A guideline[28], or its absence should be justified. When performed, safety pharmacology endpoints could be retrieved from toxicology and biodistribution combined studies.

Standard absorption / distribution / metabolism / excretion (ADME) studies are often not relevant for VV-GTMPs. Pharmacokinetics should then mainly focus on (bio)distribution, persistence, shedding and clearance of the viral vector and the transgene product and also to address any risk of germline transmission.

Toxicology studies assess the entire VV-GTMP (viral vector delivery system, transgene product) to identify any unwanted consequence of distribution / persistence of the vector, infection / transduction / transfection / expression, biological activity as well as immunogenicity or an unwanted (secondary) pharmacological effect. Any risk of off-target-tissue exposure is also to be investigated or assessed using a combination of *in silico* and *in vitro* analyses[29]. In addition, level of transgene production in both targeted and non-targeted tissues and associated potential risks, need to be evaluated[29] with dedicated endpoints related to assess the transgene expression.

Evaluation of genotoxicity, tumorigenic and oncogenic potential may be required depending on the nature of the VV-GTMP.

Reproductive / developmental toxicity, as per ICH S5(R3) guideline[30], may need to be addressed depending on product type, mechanism of action, and biodistribution results.

Although AAV vectors have been shown as poorly immunogenic, clinical studies using a VV-GTMP presented adverse effect directly linked to AAV vector doses, related to preexisting immunity against serotypes[31]. Some AAV present toxicological effects on cardiovascular function[32] and /or in liver[33] functions, based on their own serotype.

Concrete examples are provided in **Table 3** to **Table 7**, illustrating strategies followed by marketing authorization holders.

Table 3-1. Approved AAV-based (types 1, 2, and 9) GTMPs in the EU and / or US (2012-2022) as of 30 March 2023

Product name (INN) Company First date of approval (in EU or US)	Product Description Indication	Pharmacology	Biodistribution, persistence and excretion / shedding	Toxicology
Glybera (Alipogene tiparvovec) Amsterdam Molecular Therapeutics; Uniqure EU (2012) Withdrawal (2017)[34]	AAV-1 containing the human LPL gene variant LPLS447X LPLD	***In vivo*** efficacy studies in LPL deficient **mouse and cat models**	**Biodistribution and persistence** of vector DNA in **mice, rabbits, and cats**	**Single-dose toxicity studies in WT mice** (IM or IV) **Reproductive toxicity in mice** Other toxicity studies: theoretical assessment of baculoviral DNA impurity-associated toxicity
Luxturna (Voretigene Neparvovec-rzyl) Novartis Inc US[20] and EU[21] (2017)	AAV-2 containing human RPE65 cDNA IRD	***In vitro*:** cell transduction, transgene expression and protein expression in RPE65 mutant cells ***In vivo*** efficacy in **murine and canine models** of RPE65 deficiency	**Biodistribution and persistence combined to toxicity assessment,** vector DNA level up to 3 months following subretinal injection in **NHP**	**Single and repeat-dose administration in RPE65 mutant dog and normal-sighted dogs and NHP,** up to 3 months, following subretinal injection either once only, or once into each eye, and twice into the same eye
Upstaza (Eladocagene exuparvovec) PTC therapeutics Intl EU[35] (2022)	AAV-2 containing human DCC cDNA encoding for AADC protein AADC deficiency	***In vitro:*** AADC protein expression and enzymatic activity in human cell line HEK293	**Biodistribution** and **persistence** combined to **6-month toxicology study** in **rats** (bilateral infusion to the putamen), and in **NHP** following single BIP, ICV or IT administration. Evaluation of DDC DNA and mRNA expression) **Shedding** assessed in blood and CSF fluid in rats.	**Single-dose toxicity studies** in rats (30-days and 6-month observation periods) **Safety pharmacology endpoints** included in 6-month toxicity study Other toxicity studies; ADAs (anti-capsid antibodies); toxicity for poloxamer 188 included in the formulation in rats and NHP **Juvenile toxicity studies conducted in NHP** in combination with biodistribution
Zolgensma (Onasemnogene Abeparvovec) AveXis and Novartis Inc EU[23] and US[22] (2019)	AAV-9 containing cDNA of SMN gene Pediatric (<2-year) patients diagnosed with SMA	***In vivo*** relative potency assay in neonatal SMNdelta7 transgenic **mouse model** and supportive studies in spinal muscular atrophy mouse model and **healthy NHP,** following ICV and IV dosing Other ROA (IT and intracisternal) evaluated in **piglets** **Secondary PD in neonatal SMNdelta7 mice** (cardiac efficacy)	**Biodistribution and persistence** of vector DNA and human SMN transgene expression in WT **neonatal FVB / n mice** (up to 6 months, at least 18 tissues tested)	**Single dose toxicity studies combined to biodistribution assessment in WT FVB / n neonatal mice (up to 24-weeks) and NHP** (up to at least 18-months) following IV and ICV / IT administrations. Scientific literature provided to justify the lack of germline transmission

Table 3-2. Approved AAV -based (type 5) GTMPs in the EU and / or US (2022) as of 30 March 2023

Product name (INN) Company First date of approval (in EU or US)	**Product Description Indication**	**Pharmacology**	**Biodistribution, persistence and excretion / shedding**	**Toxicology**
Adstiladrin (Nadofaragene firadenovec-nrpl) Ferring Pharmaceuticals US[36] (2022)	AAV-5 vector containing human IFNα2b transgene High grade, BCG unresponsive NMIBC	Efficacy **evaluated *in vivo* in an orthotopic mouse model of human bladder cancer,** dosing of IFNα2b in urine and bladder tissue in healthy animals including rats after single and repeat administrations. Safety pharmacology of Syn3 excipient	**Biodistribution as part of the toxicity studies** conducted in **NHP** (at least 5 tissues assessed) Shedding of viral DNA and IFNα2b in urine PK of Syn3 excipient	**Repeat-dose toxicity studies in NHP** (up to 2-months observation period) and supportive toxicity study in rats, following intravesical administration. **Other toxicity studies: assessment of ADAs** (anti-AV and anti- IFNα2b antibodies), toxicity and genotoxicity of Syn3 excipient, toxicity of impurities
Hemgenix (Etranacogene dezaparvovec) CSL Behring LLC EU[37]and US (2022)	AAV-5 containing coagulation factor IX human cDNA Hemophilia B	***In vivo* studies:** efficacy (transgene expression up to 18-months after administration) in male WT and Hemophilia B **mouse models** and in male **NHP** PDDI: effect of co-medication with prednisone in WT mice	**Biodistribution and persistence combined with toxicity studies in male WT mice** (up to 18-months post administration) and in male **NHP** (up to 26-weeks post administration) following IV injection, analysis of vector DNA in 17 tissues **Shedding** of vector DNA in plasma in mice and in plasma or serum, urine, saliva and semen samples in NHP	**Single-dose toxicity in male WT mice and male NHP**, including **safety pharmacology endpoints.** **Reproductive and developmental toxicity in male WT mouse** **Genotoxicity**: integration site analysis in host genomic DNA isolated from liver tissue collected in mice and NHP **Genotoxicity:** ISA of AAV following IV injection in WT mice (20 samples) and NHP (12 samples), at 26-weeks post-dosing **Other toxicity studies: antigenicity** endpoints (anti-capsid and anti-human FIX antibodies) included as part of the general toxicity
Roctavian (Valoctocogene roxaparvovec) BioMarin Pharmaceutical EU (2022)[38]	AAV-5 containing the cDNA of the B-domain deleted SQ form of human coagulation factor VIII Hemophilia A	***In vivo* studies** using single IV administration in WT, immunodeficient Rag2$^{-/-}$ and hemophilia A/Rag2$^{-/-}$ **mouse models**, and in **NHP**	**Biodistribution and persistence** of vector DNA in WT, Rag2$^{-/-}$ and hemophilia Rag2$^{-/-}$ **mice** (up to 24-weeks) and in WT **NHP** (up to 13-weeks), following single IV administration, in at least 11 tissues Transgene expression (vector mRNA) endpoints included	**Single-dose toxicity study in WT mice** following IV administration (26-weeks observation period) Supportive safety evaluation as part of PD studies in mice and WT NHP **Germline transmission** study in mice **Genotoxicity:** ISA of AAV using TES on 12 transduced NHP liver samples **Juvenile toxicity in mice** **Other toxicity studies: ADAs** (anti-capsid antibodies); effect of concomitant chronic steroid treatment

Table 4. Approved HSV-based GTMP in the EU and / or US (2015) as of 30 March 2023

Product name (INN) Company First date of approval (in EU or US)	**Product Description Indication**	**Pharmacology**	**Biodistribution, persistence and excretion / shedding**	**Toxicology**
Imlygic (Talimogene laherparepvec, T-VEC, OncoVEX^{GM-CSF}) Amgen EU[39] and US[40] (2015)	Attenuated HSV-1 genome engineered to express hGM-CSF Melanoma regionally or distantly metastatic	***In vitro*:** comparative assessment in cell lines permissive or not for the WT HSV-1; cytotoxic effect in mouse and human (n=8) cancer cell lines. ***In vivo*** cytotoxic T cell activity and anti-tumoral effect in **human xenograft and syngeneic tumor mouse models,** in combination or not with other anti-tumoral treatments	**Biodistribution and persistence in WT or tumor-bearing mouse models,** following single or multiple SC, IV and intratumoral dosing: evaluation of viral DNA and human GM-CSF transcript levels up to 91 days post injection (13 tissues tested) **Shedding** up to 84 days post dosing in mice in excreta and shedding tissues and in **dogs** in urine (additional study)	**GLP repeat-dose toxicity studies in WT or tumor-bearing mice** (up to 12 weeks) and rats and dogs (additional studies), following SC, IV or intratumoral injection. **Developmental and reproductive studies in WT mice** **Other toxicity studies:** - **in mice:** effect of acyclovir on replication of the product, humoral response, neurovirulence following intranasal administration, effect of intracerebral administration, evaluation of virus reactivation capability in PNS - **In rats:** toxicity following intrahepatic administration

Table 5. Approved retroviral based (not LV) GTMP in the EU and / or US (2016) as of 30 March 2023

Product name (INN) Company First date of approval (in EU or US)	**Product Description Indication**	**Pharmacology**	**Biodistribution, persistence and excretion / shedding**	**Toxicology**
Strimvelis (GSK2696273) GlaxoSmithKline (GSK) EU (2016)[16]	Autologous hematopoietic stem cells modified with a RV (GSK3336223) encoding for human adenosine deaminase cDNA sequence. Adenosine deaminase-severe combined immunodeficiency	***In vitro*:** transduction efficacy (VCN) in CD34+ cells from umbilical cord blood (healthy donors) and bone marrow from adenosine deaminase-severe combined immunodeficiency patients; functional activity (adenosine deaminase protein expression) ***In vivo* engraftment following infusion in immunodeficient mice**	**Biodistribution and persistence in NSG mouse model** (VCN and adenosine deaminase protein expression) following IV administration, up to 10 weeks after transplantation.	No single-dose nor repeated-dose toxicity studies conducted (selected general toxicity endpoints included in the GLP biodistribution study conducted in mice)
Zalmoxis (Naloimagene Carmaleucel) MolMed EU[41] (2016) Withdrawal (2016)[14]	Allogenic T-cell modified with a RV encoding for a truncated form of the ΔLNGFR and the HSV-TK Mut2 GvHD in HSC transplantation	***In vitro*** (as part of batches analyses): phenotyping of transduced T cells; cell polarization (in Th1 or Th2 type) following transduction; response to ganciclovir ***In vivo*: primary pharmacology in xenograft mice models** (GvHD in NOD/SCID, and humanized animals)	**Biodistribution and persistence combined to primary pharmacology** studies in mice up to 3 weeks, using immunohistochemical assessment	**Single-dose toxicity evaluated as part of pharmacodynamic and PK evaluation in GvHD model based in NOD/SCID mouse system** (integrated approach) **Carcinogenicity: *in vitro* analyses** of the TCR repertoire (clonality of transduced cells), **cell survival** in the absence of non-dispensable growth factors **and integration profile of RV;** *in vivo* supportive cumulative data obtained from *>300 mice

Table 6. Approved genetically modified hematopoietic (CD34+) cell-based therapies in the EU and / or US (2019-2021) as of 30 March 2023

Product name (INN) Company First date of approval (in EU or US)	Product Description Indication	Pharmacology	Biodistribution, persistence and excretion / shedding	Toxicology
Zynteglo (Betibeglogene autotemcel) BlueBirds Bio EU[42] and US[43] (2019) EU withdrawal (2020)[12]	Autologous CD34+ cells transduced with functional copies of altered-globin gene using LV β-thalassemia	***In vitro*** transduction efficacy and globin chain expression in sickle cell diseases CD34+ cells and human HSC CD43+ cells ***In vivo*** evaluation of transduction effect and long-term engraftment in mice and pharmacology evaluation combined to toxicity in β-thalassemia **mouse model** following IV injection	**Cell biodistribution (VCN)** assessed in target tissues (BM and peripheral blood) **as part of combined pharmacology and toxicity studies**	**Single dose toxicity studies in β-thalassemia mouse model** (up to 6-months post-dose) **Genotoxicity:** IVIM; ISA using different lots (n=6) of LV and DNA extracted from β-thalassemia mice and WT mice bone marrow; supportive data of cell viability, cytotoxicity and VCN evaluation in mouse cells from pharmacology studies
Libmeldy (atidarsagene autotemcel, OTL-200) Orchard Therapeutics EU (2020)[44]	Autologous CD34+ HSPC transduced with a LV encoding for human ARSA cDNA MLD in children	***In vitro*:** transduction in WT and ARSA-deficient murine bone marrow-derived Lin- HPSC and healthy human CD34+ cord blood-derived HSPC ***In vivo*:** engraftment and transgene expression by murine HSPC in **WT mice** and efficacy in pre-symptomatic and symptomatic As2 -/- mice (**disease animal model**)) following IV administration **Secondary PD** to evaluate impact of ARSA overexpression on sulfatases homeostasis *in vitro* and *in vivo*	**Biodistribution and persistence** in WT and As2-/- mice with murine Lin-HSCPC transduced with GFP LV (up to 9 months post-primary injection and 3 months post-secondary injection) and in **neonate immunodeficient NGS mice** with ARSA-LV or GFP-LV transduced HSPC (up to 20 weeks post-injection) including evaluation of potential *in vivo* mobilization of ARSA LV from human to mouse cells	No single dose and repeated dose toxicity conducted. Long-term toxicity in As2-/- disease mouse model with follow-up during 12 weeks after transplantation (IV injection) **Genotoxicity: *in vitro*** immortalization, ISA after injection in Rag2-/- IL2rg-/- mice (up to 12 weeks) **Oncogenicity: *in vivo* in As2-/6 mice following IV** administration (up to 12-months observation period) **and in WT FVB mice** using GFP-LV murine Lin-HSCPC
Skysona (Elivaldoge Autotemcel) Bluebirds Bio company EU[45] and US[46] (2021) Withdrawal in EU (2021)[11]	Autologous CD34+cell genetically modified using a LV encoding for ABCD1 cDNA to express ALDP protein Early CALD (patients < 18y)	***In vitro*:** transduction efficacy (healthy and AMN human derived CD34+ cells) and production of transgenic ALDP; functional correction of VCLFA metabolism in ALD-defective primary human fibroblasts (ABCD1 mutant) ***In vivo*:** bone marrow engraftment in myeloablated **NSG mice**	**Biodistribution and persistence as part of PD and toxicity studies in NSG and NSGS** immunodeficient **mice,** following IV injection and busulfan preconditioning (for up to 3-months post administration)	**Single dose toxicity and biodistribution** studies in NSG and NSGS mice (3-months observation period) **Genotoxicity:** IVIM study; *in vitro* immortalization assay; ISA of LV in bone marrow collected from injected mice and in human CD34+ cells from healthy donors

Table 7-1. Approved CAR-T cell therapies in the EU and / or US (2017-2021) as of 30 March 2023

Product name (INN) Company First date of approval (in EU or US)	Product Description Indication	Pharmacology	Biodistribution, persistence and excretion / shedding	Toxicology
Kymriah (Tisagenlecleucel) Novartis Pharmaceuticals EU[19] and US (2017)	Autologous CAR-T cell genetically modified using LV to target CD19 (CD19-CAR-T cells) B-cell precursor ALL	***In vitro*** proliferation, cytokine production and cytotoxicity against B-ALL tumor cells ***In vivo* specificity and dose efficacy studies in leukemia mouse model**	**Biodistribution and persistence in immunodeficient mouse** engrafted with human acute B-ALL and administered CAR-T cell on day 21 (up to Day 56)	No standard single- or repeat-dose toxicity studies conducted **Genotoxicity:** ISA of the LV and toxicity for impurities and excipients in rat
Yescarta (axicabtagene ciloleucel) Kite Pharma EU[18] and US[17] (2017)	Autologous CAR-T cell genetically modified using RV to target CD19 (CD19-CAR-T cells) DLBCL	***In vitro*:** specificity, potency, poly-functionality, and cytotoxicity against primary human CLL cells Surrogate murine CD19 CAR-T cells engineered for *in vitro* nonclinical POC studies. ***In vivo*:** efficacy in a **syngeneic murine model of lymphoma** using surrogate murine CD19 CAR-T cells (including investigation of the influence of total body irradiation prior engraftments)	Biodistribution not conducted, supportive scientific literature provided. **Persistence evaluated in syngeneic mouse lymphoma model** (209-Day duration study)	No single-dose toxicity studies conducted. **On-target/off-tumor toxicity** of CD19 CAR-T **cells as part of pharmacology and persistence evaluation in mouse model** (non-GLP) Supportive scientific literature to discuss the risk of integration of RV
Tecartus (Brexucabtagene Autoleucel) Kite Pharma Inc. EU[47] and US[48](2020)	Autologous T-cell genetically modified using gamma RV to target CD19 (CD19-CAR-T cells) MCL and ALL	***In vitro*** characterization of the transduction, CAR expression and activation and expansion of CAR-T cells, activity of surrogate mouse CD19 CAR-T cells ***In vivo* studies in syngeneic mouse** lymphoma model using surrogate murine CD19 CAR-T cells	**Persistence of surrogate murine CD19 CAR-T cells in syngeneic mouse lymphoma** (up to 63 days post-engraftment)	No single- or repeat-dose toxicity studies conducted. **Genotoxicity:** ISA at the end of the manufacturing process **Other toxicity studies: on-target/off-tumor toxicity of surrogate murine CD19 CAR-T cells in the syngeneic mouse lymphoma model** in combination with persistence assessment
Abecma (Idecabtagene vicleucel) Celgene Corporation EU[49] and US[50] (2021)	Autologous T cell genetically modified using LV to target BCMA (BCMA-CAR-T cells) MM	***In vitro*:** correlation between BCMA expression, cell surface BCMA and CAR-T cells activity in human MM and lymphoma tumor cell lines and biopsies; ***In vivo*:** activity and dose response efficacy using MM cells **xenografts in NSG mice** **Secondary PD: off-target binding** of anti-BCMA antibodies and **tissue cross-reactivity** in normal human tissues	**Biodistribution and persistence** in **combination with pharmacology and safety assessment in NSG mice** liver, lung and spleen (up to 29 day)	No single dose and repeated dose toxicity conducted, safety assessed as part of the pharmacology and biodistribution studies conducted in NSG mice **Genotoxicity/carcinogenicity:** ISA of the LV on 20 patient donors; *in vitro* expansion studies (+/- IL2 cytokine)

Table 7-2. Approved CAR-T cell therapies in the EU and / or US (2022) as of 30 March 2023

Product name (INN) Company First date of approval (in EU or US)	Product Description Indication	Pharmacology	Biodistribution, persistence and excretion / shedding	Toxicology
Breyanzi (Lisocabtagene maraleucel) Juno therapeutic Inc EU[51] and US[52] (2022)	Autologous CAR-T cells genetically modified using LV to target CD-19 (CD19 CAR-T cells) DLBCL	***In vitro*:** affinity, binding specificity, cytokines production, cytotoxic activity and CAR-T cells proliferation using CD19+ target tumor cell ***In vivo*:** efficacy and dose-response effect in immunodeficient NOD/SCID IL-2Rγnull **mice engrafted with CD19+ Raji human Burkitt's' lymphoma** **Secondary PD**: tissue cross-reactivity in normal human tissues PDDI: effects of durvalumab (anti-PD-L1), CC-122 and lenalidomide immunomodulatory agents and IDO1-specific inhibitor epacadostat *in vitro*, and of BTKi (ibrutinib and acalabreutinib) *in vivo* in mice, on product activity	***In vivo* persistence** in Raji xenograft mouse model following single or repeat-dose IV administration in **Raji xenograft mouse mode**l in blood, lymphoid tissue, spleen and bone marrow, +/- cetuximab (anti-EGFR) treatment.	No single- or repeat-dose toxicity studies conducted. **Genotoxicity:** *in vitro* ISA of LV in 34 patient donors, *in vitro* CAR-T cell cytokine-independent proliferation study
Carvykti (Ciltacabtagene autoleucel) Janssen Cilag Intl EU[53] and US[54] (2022)	Autologous T cells genetically modified using LV to target BCMA (BCMA-CAR-T cells) MM	***In vitro:*** CAR-T cell characterization and binding profile; specific activation, cytokine release and cytotoxicity using BCMA+ target cells ***In vivo*:** dose escalation and efficacy studies in MM **xenograft mouse model**	Evaluation of cells **persistence and expansion** (CAR gene copy number in blood) in MM **xenograft mouse model as part of the pharmacology study** (up to 48-days duration)	**Single dose toxicity study in NHP using surrogate NHP autologous BCMA-CAR-T cells** **Genotoxicity:** ISA of LV (on 6 MM patients and 3 healthy donors) and *in vitro* cytokine-independent proliferation study Other toxicity studies: **potential off-target toxicity assessed *in vitro***

Abbreviations for Table 3 to Table 7: AADC, aromatic L-amino acid decarboxylase; ABCD1, ATP binding cassette subfamily D member 1; ALDP, adrenoleukodystrophy protein; AAV, adeno-associated virus; ADA, anti-drug antibodies; ALL, acute lymphoblastic leukemia; ALD, adrenoleukodystrophy; ARSA, arylsulfatase; AV, adenovirus; BCG, bacillus Calmette-Guerin; BCMA, B cell maturation antigen; BIP, bilateral intra-putamen; BM, bone marrow; BTKi, Bruton's tyrosine kinase inhibitor; CALD, cerebral adrenoleukodystrophy; CAR, chimeric antigen receptor; CC-122, avadomide; cDNA, complementary DNA; CLL, chronic lymphocytic leukemia; DCC, dopa decarboxylase; ΔLNGFR, truncated form of the human low affinity nerve growth factor receptor; DLBCL, diffuse large B-cell lymphoma; DNA, deoxyribonucleic acid; EGFR, epidermal growth factor receptor; h, human; GFP, green fluorescent protein; GLP, good laboratory practice; GM-CSF, granulocyte-macrophage colony-stimulating growth factor; GvHD, graft versus host disease; HSC, hematopoietic stem cells; HSPC, hematopoietic stem and progenitor cells; HSV, herpes simplex I virus; ICV, intracerebroventricular; IDO1, indoleamine 2,3-dioxygenase 1; IM, intramuscular; INN, international non-proprietary name; IRD, inherited retinal dystrophy; ISA, integration site analysis; IT, intrathecal; IV, intravenous; IVIM, *in vitro* immortalization assay; LPL, lipoprotein lipase; LPLD, lipoprotein lipase deficiency; LV, lentiviral vector; MCL, mantle cell lymphoma; MLD, metachromatic leukodystrophy; MM, multiple myeloma; NHP, non-human primate; NMIBC, non-muscle invasive bladder cancer; NOD, nonobese diabetic; NSG, NOD SCID gamma; NSGS, triple transgenic NSG-SGM3; PD-L1, program death-ligand 1; PDDI, pharmacodynamic drug-drug interaction; PK, pharmacokinetics; PNS, peripheral nervous system; POC, proof of concept; RPE65, retinal pigment epithelium 65 kDa; RV, retroviral vector; sc, subcutaneous; SCID, severe combined immunodeficiency; SMA, spinal muscular atrophy; SMN, survival of motor neuron 1; TCR, T cell receptor; TES, target enrichment sequencing; TK, thymidine kinase; VLCFA, very-long chain fatty acids; VCN, vector copy number; WT, wild type.

DISCUSSION

Tailored nonclinical approach

Nonclinical development strategies for gene therapies are different from other Medicinal Products (drugs) such as small molecules or biologics. They generally do not involve, for example, repeated-doses study but single-dose studies with a follow-up period determined on a case-by-case basis. For VV-GTMPs, doses are mostly at or slightly above the clinically efficacious dose, the limitation depends on the transgene overexpression and is influenced by numerous factors (*e.g.*, vector tropism, transgene product potency, strength of the promoter, patient population). Safety margins can also be limited by the size of the batches produced for the nonclinical development.

It is important to design nonclinical toxicology studies appropriately, as they need to address safety concerns for the patient. One major challenge of VV-GTMPs evaluation in nonclinical studies remains the duration of the studies. Indeed, the nonclinical *in vivo* studies rarely go beyond one year of evaluation (depending on the animal model), which may seem quite short when considering a therapy aimed to last for years (or for the whole life-time) in the patient. They therefore require additional *in vitro* testing, such as *in vitro* immortalization (IVIM) assay allowing quantification of the risk of vector-induced cellular transformation. Such additional testing will thus support the risk analysis prior to initiating a First-in-Human clinical trial.

From the information collected in **Table 3** to **Table 7**, considering the studies conducted to allow the authorization of 19 VV-GTMPs in the EU and / or US, it appears that no standard (or classical) nonclinical development plan could be implemented and made applicable to every gene therapy products in development (in other words, "one-size does not fit all").

Nevertheless, the following trends appear:

Pharmacology: as regards *in vitro* studies, each product should be tested according to its own properties. For example, the transduction of Libmeldy was logically tested in wild-type and ARSA-deficient murine bone marrow-derived Lin- HPSC and healthy human $CD34^+$ cord blood-derived HSPC (**Table 6**). And, when possible, the proof-of-concept *in vivo* was provided by the developers, such as for Tecartus, which was tested in a syngeneic mouse lymphoma model using surrogate murine CD19 CAR-T cells (**Table 7-1**).

As regards potential off-target effects, secondary pharmacology, studies were generally conducted to evaluate a potential risk of toxicity for other non-target tissues. Safety pharmacology endpoints, when assessed, were generally derived from biodistribution / toxicology combined studies.

Biodistribution / persistence / shedding: very consistently, the biodistribution and persistence (from a few to up to 18 tissues / organs) of VV-GTMPs was investigated, often as part of combined studies: either efficacy studies in a model of the disease in immunocompromised animals or in toxicology studies. Shedding was also generally monitored in fluids compartments, but not for all the VV-GTMPs.

Toxicology: In general, no single- or repeat-dose toxicity studies were requested by the EMA and / or FDA for CAR-T cells (Kymriah, Yescarta, Tecartus, Abecma, Breyanzi), as it was not possible to test the proposed therapies in a relevant species (**Table 7**). One noticeable exception to this "rule" is Carvykti, the BCMA-CAR-T cell developed by Janssen Cilag Intl, which was investigated as part of a single dose toxicity study in non-human primates (NHPs), using surrogate NHP autologous BCMA-CAR-T cells (**Table 7-2**). For the other VV-GTMPs (*i.e.*, not CAR-T cell products), the amount of general toxicology studies performed varied considerably. For instance, no single-dose nor repeated-dose toxicity studies were conducted with Strimvelis (**Table 5**). However, selected general toxicity endpoints were included in a GLP biodistribution study conducted in mice. On the contrary, Upstaza was tested in single-dose toxicity studies in rats (30-days and 6-month observation periods including safety pharmacology), and even juvenile toxicity studies were conducted in NHP (in combination with biodistribution assessment) (**Table 3-1**). For Zolgensma, single-dose toxicity studies were combined to biodistribution assessment in wildtype FVB/n neonatal mice (up to 24-weeks) and even NHPs were tested using three different routes of administration (**Table 3-1**).

As regards genotoxicity, IVIM (*in vitro* immortalization assays) and / or ISA (integration site analyses) were generally performed to further characterize the VV-GTMPs products. This risk was also assessed *ex vivo* (after *in vivo* administration as part of combined biodistribution / toxicology study), which was the case for Hemgenix (**Table 3-2**). In addition, for CAR-T cell products, the potential oncogenicity properties are assessed *in vitro* using cytokine-independent proliferation assay and analyzing of TCR repertoire (clonality). Generally, a risk assessment according to the literature is also requested.

Of note, reproductive toxicity studies which were deemed necessary for the most ancient approved

VV-GTMPs (Glybera and Imlygic, **Table 3-1** and **Table 4** respectively), were recently not required. Such study waiver could be granted if a biodistribution assessment is provided with evidence of absence of toxicological findings in the reproductive organs. In contrast, juvenile studies may still be demanded: e.g. Upstaza was tested in juvenile NHP in combination with biodistribution investigations (**Table 3-1**); Roctavian was tested in juvenile mice (**Table 3-2**); and Zolgensma was investigated in a neonatal model of the disease and completed with a literature-based rationale to assess a limited risk of germline transmission. (**Table 3-1**).

Another requested nonclinical activity consists in monitoring the antigenicity of the VV-GTMPs and the levels of ADAs produced (**Table 3**).

Finally, since knowledge is growing on VV-GTMPS, rationale based on literature review becomes generally acceptable to justify the lack of a study as long as the product and its mode-of-action are well-defined.

VV-GTMPs and ethics

As VV-GTMPs are intended to permanently modify the human body's genetic material, it also raises ethical concerns. One element to account for in the risk analysis is the unintended dissemination of the vector leading to the possible modification of germ line cells due to off-target – tissue effect of the VV-GTMPs.

Originally, gene therapies were designed to correct recessive monogenic defect, in bringing a healthy copy of the deficient gene in the affected cells. Associated ethical considerations should consider the following: favorable benefit / risk balance (beneficence / non-maleficence principle), informed consent (principle of respect of persons) and fairness of research subject selection (principle of justice). To date, acceptable gene therapy clinical trials involve somatic cell therapies correcting genes causing diseases. Nowadays, more genes causing different traits are discovered. Thus, there could be a "slippery slope" towards changing "comfort", "cosmetic" or "performance-associated" (e.g., genetic doping) genes to be targeted in future gene therapies. In addition, many ethicists may worry that regulation would not be ready to deal with the rapid progresses of the feasibility of germline gene therapy.

Cost is another ethical issue, impacting the accessibility of gene therapies to patients. For VV-GTMPs, pricing remains a hurdle due to the investments necessary to support manufacturing constraints and safety testing requirements. Applicants/Sponsors consider development cost, disease prevalence and related expenses (e.g., hospitalizations) and efficacy of their product to determine the price of their VV-GTMP. As shown in **Table 3-1** and **Figure 2**, Glybera was approved by the EMA in 2012. At that time, the drug was expected to cost around $1.6 million per treatment. Importantly, as of 2016, only one patient had received this drug product outside of a clinical trial[55] For this reason, in April 2017, the renewal of the marketing authorization in the European Union was not requested by the market authorization holder, due to lack of demand from healthcare providers[56] The withdrawal of several VV-GTMPs by market authorization holders based on commercial reasons, raises questions on the accessibility of efficient and approved treatments in regions where the market is not profitable. One can hope that current and future VV-GTMPs approved to safe and efficient in certain diseases remain available for all patients beyond the market authorization.

Trends for the future

This review illustrated the evolution of VV-GTMPs along with a better knowledge of the viral vectors used and the benefit/risk ratio for the patients. The approval of various VV-GTMPs for rare diseases paved the way for developing gene therapies aiming to treat clinical indications targeting larger population.

Interestingly, the full impact of gene therapy is expected to be reached soon when effective treatments for diseases with larger patient numbers appear. Yildirim and Kocabas[57] reported several examples of such evolution:

- Engensis (donaperminogene seltoplasmid) developed by Helixmith to treat diabetic peripheral neuropathy. The target population is estimated to be approximately 7.1- 13.5 million adult patients. This VV-GTMP clinical development is currently in Phase 3.
- Tavo (tavokinogene telsaplasmid), developed by Merck / Oncosec to treat advanced metastatic malignant melanoma in adult progression with checkpoint inhibitor. This product is targeting a subset of 1.2 million adult patients living with melanoma. For this product the clinical development Phase 3 is expected to be launched in 2024.
- Invossa (tonogenchoncel-L), developed by Kolon Group, is aiming to treat knee osteoarthritis using intra-articular injection. This therapy is targeting a patient population estimated at 3 million adult

patients. The confirmatory clinical development is expected to be launched in 2025.

Approved therapies are life-changing for affected patients. Now, they could be extrapolated to other conditions and patient populations, as next generation technologies are increasingly expanding[58]. In line with this observation, the former FDA commissioner, Scott Gottlieb, has predicted that by 2025, US FDA will approve 10 to 20 different gene therapies per year[59]. The market for gene therapy products is indeed expanding as the number of approved medicines grows. In 2023, cell and gene therapy products market is expected to reach $15.4 billion and may reach $34.3 billion in 2025[60].

CONCLUSION

In this review article, nonclinical development plans of 19 VV-GTMPs approved in the EU and / or US were analyzed and showed that nonclinical approaches were customized to each product and target indication (**Table 3** to **Table 7**). For example, standard standalone *in vivo* tumorigenicity or reproductive toxicity studies, which appeared relevant only a few years ago, appear outdated today and can often be substituted by either *in vitro* / *ex vivo* studies or even by literature-based rationale. Thus, stakeholders need to bear in mind that nonclinical development programs need to be individual, risk-based, and tailored to each product. Such case-by-case approach will ensure the appropriate product assessment.

In such fast-evolving environment, regulatory agencies need to adjust rapidly towards new assessment processes, taking into consideration lessons learned from the past (benchmark approach).

Conflicts of Interest Statement

CR PhD, AB, DR PhD, EV PhD are employed by Voisin Consulting Life Sciences, Boulogne (Paris), France.

CC PhD is employed by Voisin Consulting Life Sciences, Lausanne, Switzerland.

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