
REVIEW

Viral Vectors in Gene Replacement Therapy

Ekaterina Minskaia^{1,a*}, Alima Galieva¹, Alexander D. Egorov¹,
Roman Ivanov¹, and Alexander Karabelsky¹

¹Scientific Center of Translational Medicine, Department of Gene Therapy, Sirius University of Science and Technology,
354530 Sochi, Russia

^ae-mail: minskaia.es@talantiuspeh.ru

Received April 28, 2023

Revised September 29, 2023

Accepted October 17, 2023

Abstract—Throughout the years, several hundred million people with rare genetic disorders have been receiving only symptom management therapy. However, research and development efforts worldwide have led to the development of long-lasting, highly efficient, and safe gene therapy for a wide range of hereditary diseases. Improved viral vectors are now able to evade the preexisting immunity and more efficiently target and transduce therapeutically relevant cells, ensuring genome maintenance and expression of transgenes at the relevant levels. Hematological, ophthalmological, neurodegenerative, and metabolic therapeutic areas have witnessed successful treatment of hemophilia and muscular dystrophy, restoration of immune system in children with immunodeficiencies, and restoration of vision. This review focuses on three leading vector platforms of the past two decades: adeno-associated viruses (AAVs), adenoviruses (AdVs), and lentiviruses (LVs). Special attention is given to successful preclinical and clinical studies that have led to the approval of gene therapies: six AAV-based (Glybera® for lipoprotein lipase deficiency, Luxturna® for retinal dystrophy, Zolgensma® for spinal muscular atrophy, Upstaza® for AADC, Roctavian® for hemophilia A, and Hemgenix® for hemophilia B) and three LV-based (Libmeldy® for infantile metachromatic leukodystrophy, Zynteglo® for β -thalassemia, and Skysona® for ALD). The review also discusses the problems that arise in the development of gene therapy treatments, which, nevertheless, do not overshadow the successes of already developed gene therapies and the hope these treatments give to long-suffering patients and their families.

DOI: 10.1134/S0006297923120179

Keywords: gene therapy, viral vectors, hereditary disorders, gene replacement therapy

INTRODUCTION

Gene replacement therapy seeks to correct a genetic deficiency by delivering either a full-length copy or a functional part (mini-gene) of disease-causing gene to the patient's cells. Introduction of a functional gene into the patient's cells restores the protein activity and compensates for the presence of a mutant allele. Depending on the type of target cells, gene replacement therapy can be divided into two main categories: somatic and germline:

- Somatic gene therapy relies on the delivery of a functional nucleic acid into somatic cells, rather than the germ cells or gametes. While its effects are beneficial to the treated organism, they are not inherited by the offspring;

- Germline gene therapy, instead, is based on the delivery of a functional nucleic acid into gametocytes or gametes, hence the transferred gene is passed to the offspring.

Germline gene therapy is a promising approach that gives hope of eradicating the majority of hereditary diseases that are passed from one generation to another. Delivery of a functional gene copy could be performed during the early embryonic development, reproduction, or *in vitro* fertilization, when the modified gamete cells fuse to form a zygote. In these cases, the functional copy of a gene is delivered to all cells of the developing embryo.

The germline gene therapy, similarly to genetic editing of embryos, is currently illegal in more than 40 countries due to the ethical concerns and potential misuse.

Somatic gene therapy aims to deliver corrected genetic information to the tissues of an organism affected by pathology. In this case, the therapeutic nucleic acid

* To whom correspondence should be addressed.

is delivered to somatic cells, including undifferentiated stem cells. The introduced DNA either integrates into the host genome or exists as an extrachromosomal genetic element (episome). Due to the inability of somatic cells to produce gametes (sperm or eggs), the transgene is not passed to the next generation.

Somatic gene therapy utilizes two major strategies:

- *ex vivo*: cells are removed from the body, a therapeutic gene is introduced using viral or non-viral delivery tools, and the modified cells are transplanted back into the patient;

- *in vivo*: a therapeutic gene is delivered into the cells using various vector systems by intravenous (systemic) or local (*in situ*) administration.

Viral vectors are frequently chosen as delivery systems as they provide efficient distribution and expression of the therapeutic gene after a single injection, leading to a long-term response and even complete cure of patients with severe genetic disorders. During its 40-year history, gene therapy has made great strides and given hope to thousands of patients with limited or non-existent treatment options, but it has also gone through a series of setbacks associated with severe adverse effects.

The emergence of new drug development platforms based on vectors or genetically modified somatic cells has radically changed the view on traditional therapy. To date, about 3000 clinical trials of gene therapeutics have been registered, and thousands of drugs are at the preclinical trial stage [1]. Positive results from clinical studies have been demonstrated for a wide range of hereditary diseases, including hematological, immunological, ophthalmic, neurodegenerative, and metabolic disorders, as well as for several types of cancer. Gene therapy has made it possible to restore vision in patients, cure certain types of blood cancers, correct hemoglobinopathies and deficiencies of blood coagulation factors, and restore the immune system in children born with primary immunodeficiencies. The high efficacy of the first gene therapy products has accelerated their entry to the pharmaceutical market.

This review highlights three leading vector platforms of the past two decades that have been tested in preclinical and clinical studies: adeno-associated viruses (AAVs), adenoviruses (AdVs), and lentiviruses (LVs). Special attention is given not only to the successes already achieved in the gene replacement therapy using these vector systems, but also to the problems that arise in the process of developing gene therapy treatments, such as potential immunogenicity and, in some cases, lack of specificity.

AAVs: SAFER NON-INTEGRATING VECTORS FOR NON-DIVIDING CELLS

Since the first use of AAV in 1995 to deliver the cystic fibrosis transmembrane regulator (CFTR) gene to

a patient with cystic fibrosis [2], AAV vectors have been, with rare exceptions, quite successfully used in the clinic for the treatment of hemophilia [3], rheumatoid arthritis [4], blindness [5], and many other diseases. Their effectiveness and versatility are attributed to their capacity to transduce a wide variety of both proliferating and non-dividing terminally differentiated tissues [central nervous system (CNS), liver, muscle, and retina] and efficiently express transgenes for many years with an extremely low (0.1-0.5%) frequency of genomic integration [6-9].

The *rep* and *cap* genes in recombinant AAV (rAAV) vectors are replaced by a transgene and delivered by helper plasmids. The recombinant virus is produced *via* transfection of packaging cells, such as HEK293, with three plasmid DNAs: (i) plasmid encoding the transgene flanked by ITRs (inverted terminal repeats); (ii) plasmid encoding the *rep* and *cap* genes; and (iii) plasmid providing the adenoviral protein required for the AAV assembly. Unlike wild-type (wt) AAV serotype 2 (AAV2), rAAV *rep*-deficient vectors are unable to integrate into the AAVS1-specific locus on chromosome 19 [10]. After cell entry and second strand synthesis, the vector exists as an episome in nondividing cells and does not integrate into the target cell genome.

Twelve AAV serotypes and 108 new natural isolates have been identified so far: serotypes 2, 3, 5, 6 and 9 are of the human origin, while serotypes 1, 4, 7, 8, 10 and 11 were isolated from non-human primates. The serotypes differ by the mode of cell entry, tropism for tissues and receptors/co-receptors on the target cells (due to the differences in amino acid sequences typically occurring within the capsid hypervariable regions), intracellular activities, and expression patterns [11-13]. For example, AAV1 targets retina and skeletal muscles [14, 15], AAV5 efficiently transduces lung and neuronal cells [16, 17], and AAV8 is up to 100 times more efficient in liver cells compared to other tested AAV serotypes [18]. The ease with which viral capsids can be modified and optimized gives the AAV system an additional advantage.

However, application of AAV vectors has certain limitations such as (i) **slow onset of gene expression** (due to the second DNA strand synthesis); (ii) **pre-existing immunity** to the AAV capsid; and (iii) **small packaging capacity**. The step of second DNA strand synthesis can be circumvented by using self-complementary (sc) AAV vectors produced by mutating one of the two ITRs, which affects the ability of the *rep*-encoded protein to solve the replication intermediates and results in the packaging of complementary double-stranded DNA genomes. These scAAV vectors provide faster and a 10-fold higher level of transgene expression compared to single-stranded AAVs [19, 20]. They have been successfully used in clinical trials for hemophilia B [21] and spinal muscular atrophy [22].

Immune response is one of the most critical factors limiting efficient and long-term transgene ex-

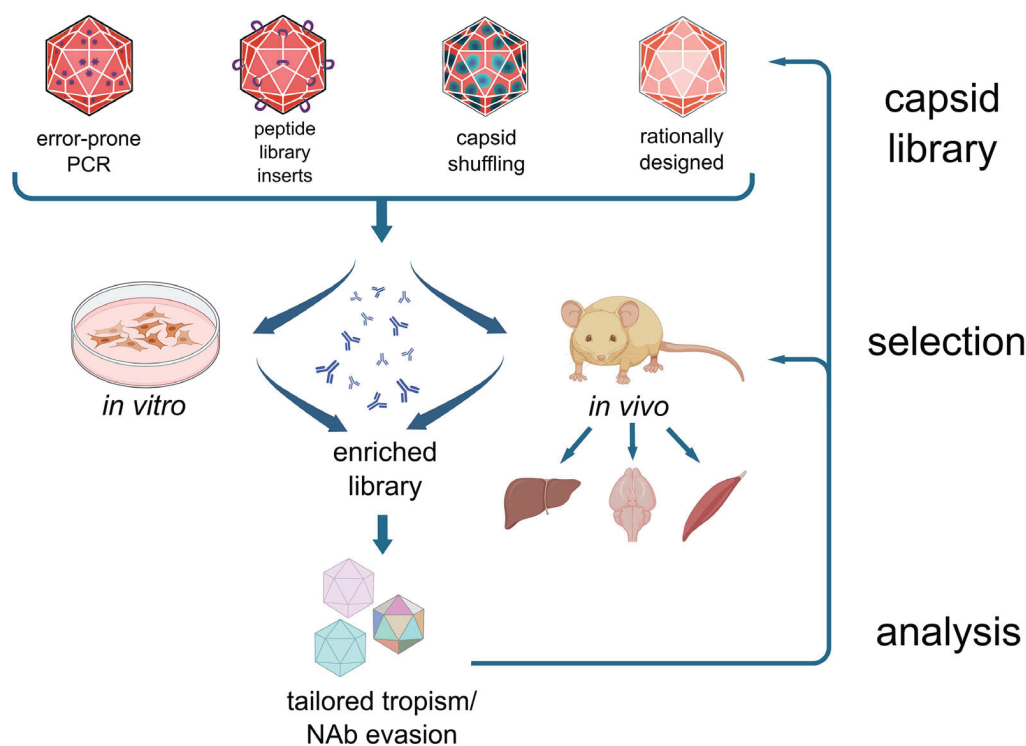


Fig. 1. Strategies of directed AAV evolution. Capsid libraries are generated by error-prone PCR (polymerase chain reaction), insertion of randomized peptides into open loop structures, capsid protein shuffling, or rational design. Selection process is based on the ability of libraries to transduce target cells or to evade nAbs.

pression [23–25]. Pre-existing neutralizing antibodies (nAbs) against the AAV capsids, whose proportion in the antibody population increases after exposure to AAV [26, 27], can efficiently neutralize rAAVs [28]. A strong immune response can also be activated by the therapeutic transgene itself [29].

Enrichment of novel synthetic AAV capsids capable of evading existing nAbs is achieved *via* two high-throughput strategies that put genetically modified AAV capsid libraries through “accelerated natural evolution”: (i) reverse genetics approach termed ancestral AAV sequence reconstruction, and (ii) AAV genome barcoding (Fig. 1). Synthetic AAV capsids include AAVrh8R, AAVrh10, AAV6.2, AAV-DJ, and Anc80 [30].

When vector platform adaptation is not possible, anti-AAV nAbs can be removed from the circulation by plasmapheresis [31]. Pretreatment with IgG-cleaving endopeptidases, e.g., imlifidase (IdeS), can reduce the content of IgG antibodies in patient’s serum [32]. For example, a homologue of IdeS isolated from *Streptococcus zooepidemicus* (IdeZ) efficiently removes anti-capsid nAbs in primates [33].

And finally, since the packaging capacity of AAV vectors is limited to ~4.7 kb, it is necessary to minimize the size of the expressed therapeutic transgene. Recombination of vectors [34], trans-splicing of RNA [35], or protein trans-splicing *via* a cleaved intein [36, 37] can be used for expression of bigger transgenes.

The development of AAV-based therapies is mainly geared towards the treatment of monogenic disorders. The first *in vivo* gene therapy drug, Glybera® (alipogene tiparvovec), approved by the European Medicines Agency (EMA) in 2012 is an AAV1-based vector that delivers an enhanced version of lipoprotein lipase (LPLS447X) gene to muscle cells. Intramuscular injections at 30–70 sites in the upper and lower extremities led to the reduction in triglyceride levels and manifestations of pancreatitis in most participants. Long-term assessment of these patients demonstrated stable and efficient expression of the therapeutic protein and a high safety of the drug [38–40]. However, Glybera® did not remain on EU market for long because of its high cost and low demand.

The treatment of previously incurable retinal diseases has become possible due to the highly efficient AAV-mediated gene transfer into the eye cells and tissues. The process of visual phototransduction is disrupted in patients with Leber’s congenital amaurosis type 2 (LCA2) due to mutations affecting expression of functional 65-kilodalton retinal pigment epithelial protein (RPE65). Three independent clinical studies with more than 30 participants demonstrated that AAV2-mediated subretinal delivery of the therapeutic RPE65 gene improved vision for at least 3 years. The second dose of the vector, received by three patients in the other eye, improved retinal and visual functions with no immune

response [41–49]. Significant improvement of the visual function was also observed in pediatric patients [50]. LCA2 became the first hereditary retinal disease for which the use of AAV vectors demonstrated impressive results [42–44, 51]. The AAV2-based drug Luxturna® (voretigene neparvovec-rzyl) delivering RPE65 gene was approved for commercial use in 2017 [5].

Encouraging results were obtained in the clinical trial for choroideremia (NCT01461213), a type of complete blindness resulting from the slow and progressive degeneration of photoreceptors, choroid, and retinal pigment epithelium [52]. Subretinal injections to 14 participants of the AAV2 vector delivering Rab-escort protein 1 (REP1) significantly improved vision in two patients and increased retinal sensitivity in four other patients after six months of therapy.

Two clinical trials used AAV2 expressing an anti-VEGF protein for the treatment of neovascular age-related macular degeneration (AMD) by subretinal (NCT01494805, 40 participants) or intravitreal (NCT01024998, 19 participants) injections. The third tested therapy, ADVDM-022 (NCT03748784, 30 participants), relied on the AAV7-mediated delivery of VEGF-binding protein (aflibercept) that blocked the growth and abnormal vascular permeability associated with wet AMD and diabetic retinopathy. Intravitreal injections of this vector led to the improvement in mean corrected vision and visual acuity and decrease in the thickness of the central subfield of the macula [53].

AAV was also used to treat Duchenne muscular dystrophy (DMD), an X-linked neuromuscular genetic disease that affects 1 in 3500–5000 males worldwide. DMD is caused by mutations in dystrophin, one of the largest proteins in human body. The discovery of a truncated form of this protein (microdystrophin) in the studies of Becker muscular dystrophy has led to the development of SRP-9001 (delandistrogene moxeparvovec) therapy [54]. Microdystrophin can be easily packaged together with striated muscle-specific α MHCK7 regulatory cassettes containing α -myosin combined heavy chain enhancer and creatine kinase version 7 promoter [55]. Forty-one participants were treated by systemic delivery of SRP-9001 to skeletal and cardiac muscles in clinical trial (NCT03769116). In another clinical trial (NCT03368742), SGT-001 infused intravenously provided AAV9 vector-mediated expression of microdystrophin. Although the results of the trial involving 16 participants showed that 50–70% of muscle fibers expressed the therapeutic transgene leading to the reduction of muscle damage, some patients demonstrated side effects, with one patient developing immune response [56]. Another type of muscular dystrophy, limb-girdle muscular dystrophy (LGMD), was treated with SRP-9003 (bidridistrogene xeboparvovec), which delivers β -sarcoglycan (NCT03652259). The absence of β -sarcoglycan causes type 2E LGMD.

Zolgensma® (onasemnogene abeparvovec by Novartis), the third AAV-based therapy developed for the treatment of spinal muscular atrophy (SMA), has been approved in 45 countries, including US and Japan in 2019 and Russia in 2022, with more than 2500 patients treated globally [22]. Zolgensma® provides the scAAV-driven expression of *SMN1* (survival of motor neuron 1) gene in motor neurons to replace dysfunctional mutated *SMN1* in patients with SMA. At the time of market launch, the treatment cost over 2 million dollars per dose.

Hemophilia is a life-threatening hematological disease caused by mutations in coagulation factors VIII (hemophilia A) or IX (hemophilia B) that occurs in 1 in 5000–30,000 males worldwide. In its severe form (<1% coagulation factor activity), hemophilia causes spontaneous bleeding into joints and soft tissues. Current treatment is extremely expensive and relies on twice/thrice-weekly intravenous infusions of either recombinant or plasma-derived FVIII/FIX proteins. Protein expression even at 5% of the normal level has a significant effect on the frequency of bleeding. FVIII is mainly produced in endothelial cells, while FIX is synthesized in hepatocytes. Initial clinical trials demonstrated significant improvements in patients with hemophilia B following AAV2-mediated gene transfer [3, 57, 58]. High doses of the drug (2×10^{12} gv/kg) resulted in FIX expression for more than 2 months with the peak activity levels of about 12%. Unfortunately, the cytotoxic T cell response to the AAV capsid halted FIX expression and led to a mild dose-dependent liver toxicity and asymptomatic elevation of transaminases [3, 58, 59]. Further studies revealed the activity of CD8⁺ memory T cells against the capsid, which may have attacked transduced hepatocytes. The second clinical trial combined immunosuppressive prednisolone treatment with the AAV8-based delivery of the codon-optimized FIX sequence [20, 60]. Switching to a different AAV serotype lowered the frequency of nAbs and made the therapy available to more patients. In preclinical trials, similar vector doses (up to 3×10^{12} gv/kg) sustained expression and improved the catalytic activity of FIX-Padua in animal models of hemophilia B [3, 61–64]. In phase I/II clinical trial (NCT01687608) with 30 participants, expression of naturally occurring hyperactive FIX variant (R338L, FIX-Padua) was achieved using scAAV8 vector. One patient who received an average dose of 1×10^{12} gv/kg demonstrated stable protein expression at a level of 20–25% of normal expression [65, 66]. While patients in the medium dosage group showed transient protein expression in the absence of hepatotoxicity and immune response, those in the highest dose group lost the transgene expression and developed IFN- γ -producing T cells in response to the AAV capsid even in a combination with prednisolone regimen. In other trials, more than 20 participants treated with various AAV-FIX vectors did not develop an immune response against

the FIX protein. In these trials, the AAV2 and AAV8 vectors were administered intramuscularly (AAV2), intra-hepatically (AAV2), or intravenously (AAV8) [3, 21, 58]. Sixty-eight clinical trials are currently testing the efficacy of various gene therapies for the treatment of hemophilia A and B. To date, successful therapies include SPK-8011 (Spark200) and Roctavian® (formerly known as Valrox or valoctocogen roxaparvovec/BMN 270) for hemophilia A and SPK-9001 (fidanacogen elaparvovec), Hemgenix® (formerly known as AMT-061 or EtranaDez), and FLT180a (verbrinacogene setparvovec) for hemophilia B. The results of clinical trials included the lack of need for enzyme replacement therapy (ERT) for 2 years or more in most patients, significant reduction in the frequency of spontaneous bleeding, and restoration of plasma levels of coagulation factors after a single injection, leading to the approval of two AAV5-based products, Roctavian® [67] and Hemgenix®, in 2022. As it was mentioned above, the use of capsid variants other than AAV2 (the most common natural AAV serotype) may enhance the transduction ability and decrease the immune response.

Non-monogenic disorders can also benefit from AAV gene therapy. For example, AAV1 vectors expressing SERCA2a (sarcoplasmic reticulum calcium ATPase) were delivered intracoronarily for the treatment of heart failure (NCT02346422), resulting in the improvement in the functional status and left ventricular function and the presence of specific biomarkers in almost all patients [68, 69]. Phase II trial (NCT01966887) also showed reduction in the incidence of cardiovascular events and decrease in the average duration of hospitalization one year after the therapy [68].

In several clinical trials, AAV vectors have been utilized for the treatment of the **Parkinson's disease (PD)**. Thus, the AAV2-mediated delivery of glutamic acid decarboxylase (GAD) into the subthalamic nucleus resulted in significant improvements in the motor function scores for at least 12 months post treatment and in a significant decrease in thalamic metabolism [70, 71]. In the phase II trial (NCT00643890), the treated group demonstrated a significantly greater improvement in the UPDRS (unified Parkinson's disease rating scale) motor performance compared to the control group during the 6-month follow-up period [71].

Aromatic L-amino acid decarboxylase (AADC) deficiency affects the synthesis and secretion of neurotransmitters, such as dopamine and serotonin, leading to the developmental delay, dystonia, hypotension, severe motor impairment, tongue protrusion, jaw spasms, and neurological impairment in infants. Patients affected by this disorder normally live for about 5 years due to severe motor dysfunction. Interestingly, Taiwanese have a high prevalence of AADC deficiency due to the founder mutation IVS6+4 A>T. In the phase I/II clinical trial (NCT01395641), the AAV2 vector carrying the AADC

gene was injected into the midbrain region of the patients [72, 73]. The patients gained weight 3-6 months post gene therapy, had better head control, and demonstrated emotional stability and improved motor and cognitive functions within 15-24 months of gene therapy [74]. Out of ten participants, three could sit with support, four could stand with support, and none experienced virus-associated toxicity. The success of clinical trials (NCT01395641 and NCT02926066) led to the registration of Upstaza® (eladocogene exuparvovec) in Europe in 2022.

CLINICAL LANDSCAPE OF AAV VECTORS

At present, the major therapeutic areas using AAV vectors include treatment of ophthalmological (26%), neurological (21%), metabolic (18%), hematological (15%), and musculoskeletal (9%) disorders (Fig. 2a, Table S1 in the Online Resource 1). In the last twenty years, the total number of AAV-based therapies has increased in all therapeutic areas but especially in the treatment of ophthalmological and metabolic disorders (Fig. 2b). Therapies directed at the treatment of ophthalmological disorders require low average dose (2.9×10^{11} gv/kg), have the highest efficacy, and demonstrate a good safety profile with fewest treatment-emergent serious adverse events (TESAEs).

Metabolic disorders are caused by the functional deficiency of proteins involved in metabolic regulation, which leads to a widespread tissue damage, multi-organ dysfunction at the early age, and often death [75]. To achieve a necessary effect, patient treatment requires high average doses (1.85×10^{13} gv/kg) and, therefore, has a relatively low safety profile. The two other major areas that have seen a rise in the number of clinical trials in the last ten years are neurology and hematology.

Twenty-nine percent of AAV-based therapies reported meeting the primary efficacy endpoint (Fig. 3a) and 44% met the safety requirements (Fig. 3b, Table S1 in the Online Resource 1). The highest efficacy (90%) was achieved in the treatment of musculoskeletal disorders which, unfortunately, had the lowest safety profile, as it requires the highest dose (9.1×10^{13} gv/kg) compared to other therapeutic areas because of systemic administration needed for correction of muscle cells of the entire body [76].

Intravenous administration is the most common delivery route (38%, Fig. 4a, Table S1 in the Online Resource 1), the use of which has significantly increased in the last 10 years (Fig. 4b). It is followed by subretinal (17%) and intracranial routes (15%) due to the rise in the number of clinical trials in ophthalmology and neurology.

While the highest efficacy (80%) demonstrated in clinical trials has been achieved using intravenous administration (Fig. 5a), this delivery route demonstrated low

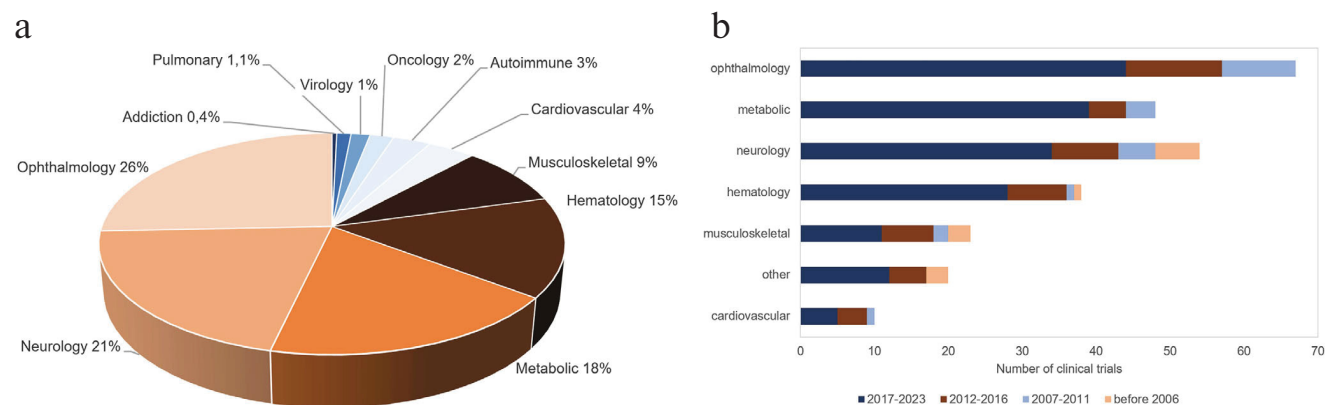


Fig. 2. AAV-based clinical trials by therapeutic area (a) and by year (b). The category “other” includes autoimmune, infectious, oncological, and pulmonary diseases.

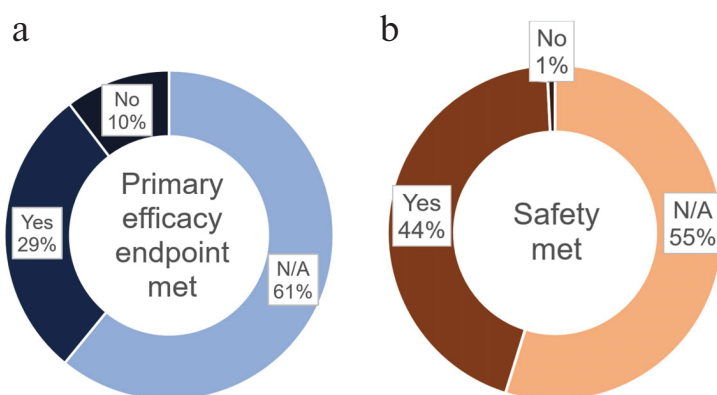


Fig. 3. Efficacy (a) and safety (b) of AAV-based gene therapies.

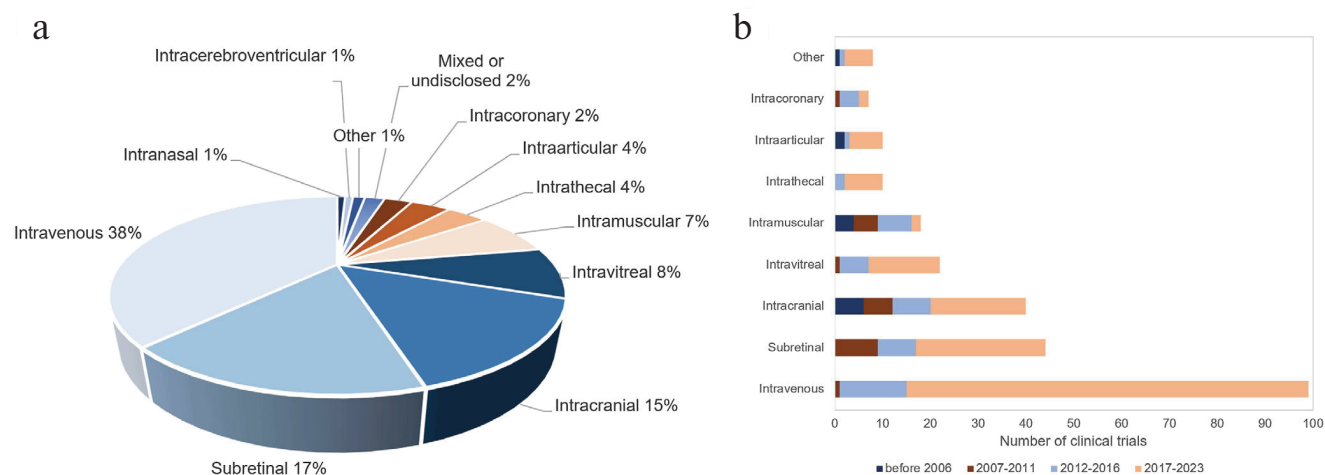


Fig. 4. AAV-based clinical trials by administration route (a) and by year (b).

safety profile with 7 clinical trials (29%) not meeting the safety requirements (Fig. 5b), which can be explained by the highest average dose (4.4×10^{13} gv/kg) required for achieving the therapeutic success [77]. Importantly, 79% of all clinical trials have demonstrated high safety profile without TESAEs, and those using subretinal (77%) and intramuscular (70%) administration routes

have demonstrated both high success rates and safety. Noteworthy, systemic and subretinal routes of administration, which require higher doses, have greatly benefited from the improvements at different stages of virus production, such as improved virus purification protocols and use of suspension producer cells and stable cell lines as an alternative to transient transfection [78].

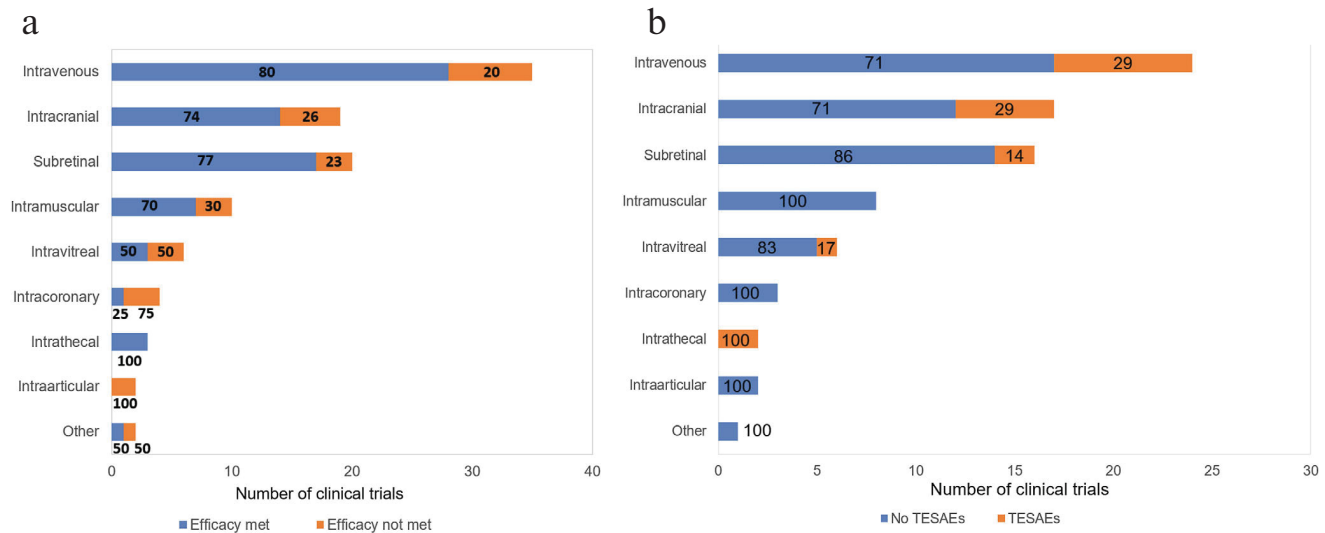


Fig. 5. Efficacy (a, $n = 101$) and safety (b, $n = 79$) of AAV-based clinical trials by route of administration.

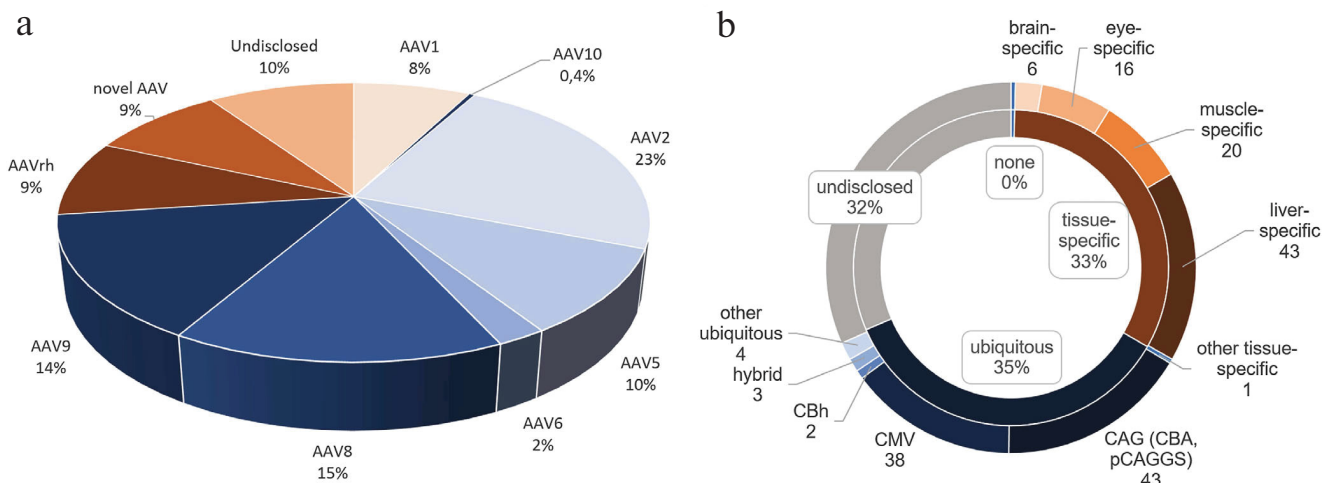


Fig. 6. AAV capsids (a) and promoters (b) used in clinical trials. CBA/CBh, chicken beta-actin promoter; CMV, cytomegalovirus promoter.

Among AAV serotypes, AAV2 (23%), AAV8 (15%), AAV9 (14%), and AAV5 (10%) are the most frequently used (Fig. 6a, Table S1 in the Online Resource 1), with AAV5 and AAV9 demonstrating the highest efficacy during clinical trials in the last 5 years followed by AAV8.

This can be attributed to the increase in therapies directed at the treatment of neurological (serotypes AAV5 and AAV9) and liver disorders (AAV8) [79]. Unfortunately, AAV9-based therapies caused the highest incidence of side effects compared to other serotypes, which can be due to systemic administration requiring high vector dose, off-target transduction, and elevated genotoxicity [80,81].

The highest success rates (80%) have been achieved in clinical trials based on new AAV serotypes, while naturally occurring serotypes AAV1 and AAV2 have been the least successful due to the presence of nAbs in 70% of the population [62, 63] resulting in low viral trans-

duction efficiency [3, 64]. Popular synthetic AAV capsids include AAVrh8R, AAVrh10, AAV6.2, AAV-DJ, and Anc80 [30, 82]. For example, AAVrh10 was used to deliver hAPOE2 (apolipoprotein E) to treat Alzheimer's disease (NCT03634007), AAVrh74 – to deliver β -sarcoglycan to treat type 2E LGMD (NCT03652259), and AAV4D-C102 – to deliver hGLA (galactosidase A) to treat Fabry disease (NCT04519749).

Constitutive promoters, such as CMV (cytomegalovirus) and chicken beta-actin (CAG) promoters, provide strong transgene expression but bear high risks of inactivation [83–85], overexpression-related toxicity [86–88], off-target expression [89, 90], and strong immune response compared to tissue-specific promoters [91, 92]. Nevertheless, 35% of clinical trials relied on ubiquitous promoters, with CMV used in 38% and CAG in 43% of these trials (Fig. 6b, Table S1 in the Online Resource 1). Acute photoreceptor and retinal pigment epithelium

toxicity in response to AAV vectors with constitutive promoters CAG, CMV, and UbiC (but not with photoreceptor-specific promoters) was observed in [93]. Tissue specific promoters, such as liver-, muscle-, eye-, and neuron-specific, demonstrated both improved safety profiles and the highest success rates compared to CMV and CAG promoters [94]. Thirty-three percent of clinical trials used vectors with tissue-specific promoters. Liver-specific promoters (43% of studies), such as LP1 in AAV5-hFIXPadua and scAAV2/8-LP1-h FIXco for hemophilia B treatment, have been successfully delivered systemically, while eye-specific promoters (16% of trials) driving expression of RPGR (for retinitis pigmentosa) and RPE65 (for LCA2) *via* AAV 2/5 were administered subretinally and intravitreally, respectively [95, 96]. Muscle-specific (20% of trials) and brain-specific (6% of trials) promoters have been successfully used for the targeted transgene expression in muscle and brain cells.

ADENOVIRUSES

Adenoviruses (AdVs), which are represented by more than 50 serotypes, were one of the first viral vectors used for *in vivo* gene therapy [97–99].

Three generations of AdV vectors have been engineered for improved therapeutic performance: (i) removal of the E1 and E3 subunits increased the packaging capacity of the first-generation AdV vectors to about 7.5 kb [99, 100]; (ii) removal of the E1 and E4 subunits significantly reduced the immunogenicity of the second-generation AdV vectors [101]; while (iii) deletion of all viral genes increased the packaging capacity of the third-generation vectors to about 30 kb [102].

AdV vectors based on human AdV2 and AdV5 serotypes have not found wide application in gene replacement therapy and, instead, are used mostly for combating cancer, production of vaccines against infectious diseases, and expression of immunostimulatory and apoptotic genes.

The advantages of these vectors include:

- high transduction efficiency in both non-dividing and dividing cells;
- broad tissue tropism;
- high packaging capacity (~36 kb);
- epichromosomal persistence in host cells;
- lack of integration into the host cell genome;
- availability of scalable manufacturing technologies [103].

AdV vectors were used for the expression of α -1 antitrypsin (A1AT) in rat hepatocytes and lungs [104, 105], CFTR (cystic fibrosis transmembrane conductance regulator) in lung tissues of patients with cystic fibrosis, and VEGF (vascular endothelial growth factor) in patients with peripheral vascular diseases [106, 107]. How-

ever, the death of a young patient with ornithine transcarbamylase deficiency due to systemic inflammation and multiple organ failure has negatively affected the research and development of gene therapy [108].

Although these viruses cause mild respiratory disease or conjunctivitis in healthy adults, several studies have shown that AdV vectors are highly immunogenic [109, 110]. Pre-existing immunity to the virus, emergence of capsid-induced immune response, and adaptive immune response to *de novo* synthesized viruses and transgenes raise safety concerns and constitute key challenges in the development of AdV vectors [97, 111]. Utilization of either rare human serotypes with low seroprevalence (hAd2, hAd26, and hAd35) or various non-human AdV vectors (bovine, canine, chimpanzee, sheep, pig, and avian) helps overcome these issues.

AdVs have been used to deliver immunogenic factors for cancer prevention. Immunization against malignant cells induces expression of tumor-associated antigens and promotes antitumor immunity. The tested antigens include MAGE-A3 for solid tumors (NCT02285816), lung cancer (NCT02879760) and melanoma/cutaneous squamous cell carcinoma (NCT03773744), prostate specific antigen for prostate cancer (NCT00583752 and NCT00583024), and cancer embryonic antigen (CEA) for colorectal and pancreatic cancers (NCT03329248 and NCT03387098, respectively).

γ -RETROVIRUSES: “TEMPERAMENTAL” INTEGRATING VECTORS FOR DIVIDING CELLS

Retroviral vectors have been extensively used for the treatment of monogenic disorders due to several advantages such as (i) stable expression of the transgene as a result of integration into the host cell genome, (ii) large packaging capacity of about 9–12 kb, (iii) efficient transduction of mammalian cells both *in vitro* and *in vivo*, and (iv) low immunogenicity [112, 113]. One of the great advantages of these viruses, namely, integration into the host cell genome, also presents a concerning disadvantage as it bears the risk of insertional mutagenesis at the integration site caused by either disruption or incorrect activation of transcription of the adjacent genes. This unfortunate event has occurred in the X-SCID (X-linked severe combined immunodeficiency) clinical trials [114, 115]. The inability to transduce non-dividing cells, lower titers compared to other viral vectors, and low replication efficiency of self-inactivating (SIN) vectors also present serious concerns when choosing these vectors for gene replacement therapy [116].

Primary immunodeficiencies (PIDs), such as X-SCID, ADA-SCID (severe combined immunodeficiency with adenosine deaminase deficiency), chronic granulomatous disease, and Wiskott–Aldrich syndrome,

are rare life-threatening genetic disorders that have a detrimental effect on the immune system. Children with severe PIDs are unable to fight off pathogens that are harmless to healthy, immunocompetent individuals, and suffer from recurrent infections (demonstrating susceptibility to certain pathogens), exhibit developmental delays, and without successful bone marrow transplantation, die within the first few years of their lives. Hematopoietic stem cell (HSC) transplantation is only available to about 20% of patients due to the lack of HLA (human leukocyte antigen)-matched donors [117]. The development of autologous HSC-based gene therapy has opened new opportunities for the patient treatment in the absence of compatible donors and without immunosuppressive regimen to prevent the graft-versus-host disease (GVHD) [9, 118-122].

The first clinical trials for the treatment of **ADA-SCID** [120, 121, 123-125] and **X-SCID** [126] relied on *in vitro* transduction of HSCs with γ -retroviral MLV vectors encoding the γ c gene (γ -chain of interleukin-2 receptor, IL2RG) or the ADA gene [120, 121, 126]. Although ADA-SCID patients with the mutant enzyme adenosine deaminase (ADA) responsible for the elimination of toxic purine metabolites from the body, experienced some beneficial effects of this therapy, the overall results of clinical trial demonstrated the need for higher numbers of engrafted modified HSCs. It is worth mentioning that patients were also receiving ERT, which complicated interpretation of the effects of gene therapy. Successful reconstitution of immune system (without the need for ERT or protective environment) was observed in eight out of 10 participants of the second ADA-SCID clinical trial [120, 121]. In subsequent clinical studies, the patients were preconditioned with a non-myeloablative regimen and ERT was discontinued prior to the infusion of autologous HSCs expressing functional ADA enzyme [121, 127-129]. Physiologically relevant ADA levels and restoration of immune function were observed in several immune cell populations. Importantly, more than 40 participants did not develop viral vector-related genotoxicity. These results were very encouraging as without gene therapy, the life expectancy of these patients does not extend beyond infancy. Strimvelis®, the first *ex vivo* ADA-SCID gene therapy product, was approved in Europe in 2016.

X-SCID is characterized by the lack of T-cells, dysfunctional B-cells, and the absence of the development of natural killer (NK) cells. The results of X-SCID gene therapy supported the idea that modified cells have a selective advantage and can effectively restore immune function in patients. A clinical trial for the X-SCID treatment showed the recovery of immunity in 18 out of 20 patients without the need for ongoing ERT and protective environment. In patients who had received the modified HSCs, T/B cells and NK cells expressing γ c transgenes were detected 10 months post treatment

and their numbers and functional activity were comparable to those in the healthy control group [126]. Unfortunately, five out of 20 patients developed clonal T-cell leukemia as a consequence of vector integration near the proto-oncogene locus, four of these patients were receiving standard chemotherapy [115, 130-132]. Despite the genotoxicity-associated side effects, the results of these trials exceeded expectations and outcomes of conventional treatment and demonstrated that the benefits of gene therapy far outweigh its risks.

Interestingly, analysis of modified cells in X-SCID patients who had developed leukemia demonstrated that integration occurred near the LMO2 gene locus, near the BMI1 and CCND2 proto-oncogenes [114, 115, 131, 132]. It was shown that the retrovirus integrated into the host genome pseudo-randomly, preferring actively transcribed DNA regions (near gene transcription start sites, regulatory elements, CpG islands), as well as conserved non-coding sequences and “hot spots” enriched in growth-controlling genes and proto-oncogenes [133-136]. The use of self-inactivating vectors lacking the long terminal repeat (LTR) enhancer or promoter reduced insertional mutagenesis and clonal expansion [137-139]. Phase I/II clinical trials for the X-SCID treatment (NCT01410019, Paris; NCT01175239, London; and NCT01129544, USA) used a SIN γ -retroviral vector expressing a correct copy of the IL2RG gene for transduction of autologous HSCs [140]. The outcome of these trials was restoration of the T cell populations in most patients, leading to their ability to fight off infections. Importantly, no adverse events associated with the insertional mutagenesis had been reported during the 3-year follow-up period, thus demonstrating the efficacy of these vectors. The observed speed of immune recovery was an additional evidence confirming the superiority of this approach over conventional haploidentical HSC transplantation [141]. While SIN-MLV vectors have reduced transduction efficiency, they do not integrate into the growth-related genes and transcription start sites compared to their conventional counterparts [142, 143]. This and related strategies, such as the use of chromatin insulators or alternative promoters, can lead to the development of new MLV vectors with improved genotoxicity profiles [114].

Retroviral vectors have also been used to treat the **Wiskott–Aldrich syndrome (WAS)**, a rare PID characterized by suppressed immune function in boys and reduced ability to form functional platelets due to the loss-of-function mutations in the WAS protein (WASp). In 2006-2009, 10 patients received autologous HSCs transduced with a retrovirus – carrying a copy of the WAS gene following a mild myeloablation regimen [144, 145]. Successfully engrafted modified HSCs demonstrated proliferative and selective advantage over unmodified cells. The level of the therapeutic protein in these patients correlated with the improved clinical manifestations,

such as partial to complete elimination of bleeding, eczema, immunodeficiency, and autoimmunity. However, 1 to 5 years after the gene therapy, seven out of ten patients developed leukemia leading to the death of two patients.

LENTIVIRUSES: INTEGRATING VECTORS WITH LOW GENOTOXICITY

In 1996, human immunodeficiency virus (HIV) was first used as a lentiviral vector (LV) to efficiently deliver a transgene [146]. LV vectors have four important features that make them suitable for therapeutic transgene delivery: (i) ability to transduce both dividing and non-dividing cells; (ii) integration into the host cell genome, which ensures long-term transgene expression; (iii) high packaging capacity (up to 9 kb) allowing expression of multiple therapeutic genes [147-149]; and (iv) relatively low immunogenicity [150, 151]. Transgene expression from LV vectors can be regulated by using inducible promoters [152] and improved by utilizing endogenous miRNAs for posttranscriptional regulation, thus dampening the transgene-induced immune response [153]. Transduction efficiency can be improved by using LVs pseudotyped with simian immunodeficiency virus and Ebola virus glycoproteins, as was demonstrated for transduction of HSCs and retina [154].

LV vectors have become invaluable for *ex vivo* transgene delivery due to their reduced genotoxicity compared to γ -retroviral vectors [135, 139, 155]. More than a dozen completed clinical trials based on LV vectors had been carried out to treat a variety of diseases, including metabolic disorders, cancer, and immune disorders. The number of ongoing trials has increased significantly due to the LV safety profile compared to other retroviruses.

One of PIDs treated by the LV-based gene therapy is **β -thalassemia**, a disorder characterized by the loss of functional β -globin (an essential component of erythrocyte hemoglobin), which result in premature death in the absence of treatment. The maintenance therapy for patients with this disorder, consisting of frequent blood transfusions and chelation therapy to prevent iron accumulation, improves the quality and duration of life but has various side effects. The first successful treatment for β -thalassemia relied on the infusion of autologous HSCs transduced with the β -globin-encoding SIN-LV, following a myeloablative regimen. As a result of therapy, patients did not require blood transfusions and their hemoglobin levels remained stable for almost 2 years. The treatment was especially successful in patients with the β^E type β -thalassemia who had some level of endogenous β -globin expression, while only partial correction was observed in patients with the β^0/β^0 type β -thal-

assemia who completely lacked the protein. Almost 3 years after the gene therapy, a patient with the type β^E β -thalassemia maintained hemoglobin levels comparable to those of healthy adults and did not require blood transfusion and iron chelation therapy, which significantly improved the life quality [156, 157]. The gene therapy product Zynteglo® (LentiGlobinBB305) for the treatment of β -thalassemia (based on clinical trials NCT03207009 and NCT02906202) was approved in 2022.

In patients with **sickle cell anemia**, red blood cells look sickle-shaped, lack flexibility, and stick to the vessel walls as a result of mutations in β -globin leading to the production of an abnormal form of hemoglobin called hemoglobin S. The blockage of blood vessels slows or stops blood flow, prevents oxygen from reaching nearby tissues, and causes pain. Transduction of HSCs with the LV vector expressing the therapeutic β -globin gene with the missense mutation β^A -T87Q was successfully performed in a 13-year-old patient who was not responsive to conventional treatment [157, 158]. As a result, the patient no longer required blood transfusions, as the levels of β -globin expression reached 47%. Phase III clinical trial for the treatment of sickle cell anemia (NCT04293185) is in the active phase and estimated to complete in 2027.

It was also shown that infusion of modified HSCs expressing the wild-type lysosomal enzyme can positively influence the fatal CNS neurodegenerative disorders X-linked **adrenoleukodystrophy (ALD)** caused by mutations in the *ABCD1* gene and characterized by the accumulation of long-chain fatty acids that damage myelin. This disease usually has a very poor prognosis in the absence of allogeneic HSC transplantation. In one of the first ALD trials, two patients were administered HSCs transduced with the LV pseudotyped with the vesicular stomatitis virus (VSV-G) glycoprotein SIN G and expressing the *ABCD1* gene. Demyelination in the brain stopped 14 months after the therapy in 1 patient and 20 months after the therapy in the second patient, and no new lesions were observed in both patients 36 months post therapy [159]. The success of phase I/II (NCT01896102) and phase III (NCT03852498) clinical trials culminated in the registration of the LV-based product Skysona® for the treatment of cerebral ALD in 2022.

Twenty-nine out of thirty pediatric ADA-SCID patients did not require ERT as a result of clinical trial carried out at two US clinical centers that used autologous HSCs transduced with the LV vectors expressing the ADA transgene [160]. Following this success, the drug OTL-101 entered phase III clinical trial (NCT04140539). SIN LVs with the cell-specific promoter and codon-optimized ADA gene were also successfully used in two clinical trials (NCT01380990 and NCT01852071) [161, 162] following non-myeloablative

regimen with busulfan. A significant increase in the total T cell counts and overall restoration of immune function were observed during the 36 months post treatment.

Based on preclinical studies, SIN-LVs for the treatment of WAS (NCT01515462) have been developed. Three participants in the phase I/II clinical trial were administered with *ex vivo* modified HSCs transduced with the LV-w1.6W vector expressing wild-type WASp gene, resulting in reliable, stable, and long-term HSC engraftment, as well as improved T cell function. In addition to the stable WASp protein levels, significant improvement in the clinical symptoms (eczema and recurrent infections) and reduced disease severity were observed at 6 months post gene therapy. Importantly, no insertional mutagenesis or clonal expansion were detected within 18–32 months after transplantation of modified HSCs, confirming the safety and efficacy of used LV vectors [163]. Encouraging results were also demonstrated in a group of patients participating in a similar gene therapy trial [164, 165].

Infantile metachromatic leukodystrophy is a fatal genetic disorder with the symptoms appearing in the second year of life and leading to the fatal outcome within the first 10 years of life. In this disorder, demyelination of the nervous system leads to severe motor and cognitive impairments caused by the lack of arylsulfatase A (ARSA), which results in the accumulation of sulfatide in myelin-producing cells. Unfortunately, conventional bone marrow transplantation does not improve the outcome of this disease and generally does not prevent the loss of motor function. In an attempt to cure this condition, presymptomatic children were infused with autologous SIN LV-modified HSCs delivering ARSA to the CNS following busulfan treatment (NCT01560182) [122]. Therapeutic levels of functional ARSA protein were detected two years post therapy; the participants demonstrated normal for their age motor and cognitive development, and, critically, no insertional mutagenesis was observed [159]. Following the success of this therapy, the LV-based product for the ARSA gene delivery to HSCs, Libmeldy®, was registered in Europe in 2020.

APPROVED GENE THERAPIES

Excitingly, sixteen viral vector-based gene replacement therapy products have been approved in the United States, European Union, and Russia, but their clinical investigation is still expanding [166].

Several **anti-cancer** gene therapy products have been approved so far. For example, **Imlygic®**, the first approved product for the treatment of melanoma, is based on a modified HSV1 expressing human granulocyte-macrophage colony-stimulating factor (GM-CSF) gene in place of the viral genes γ 34.5 and α 47 that encode ICP34.5 (infected cell protein 34.5) and ICP47,

respectively [167]. The recombinant virus can selectively replicate in malignant tissues due to the lack of ICP34.5, while the lack of ICP47 activates the host immune response. Targeted expression of GM-CSF causes infiltration of dendritic cells and leads to the development of adaptive immune response against malignant cells [167]. The efficacy of Imlygic® against a variety of cancers, such as ovarian, pancreatic, and rectal cancers, soft tissue sarcoma, and many others, is currently tested.

Gendicine®, the first AdV-based anti-cancer gene product, was approved in China in 2003 for treating head and neck squamous cell carcinoma [168]. It is based on human AdV5 in which the E1 gene was replaced by the gene encoding the pro-apoptotic p53 protein (human tumor suppressor) that blocks the cell survival pathways. Thousands of patients have been treated with Gendicine®, who demonstrated a significantly better response when the gene therapy treatment was combined with chemotherapy, radiotherapy, and other conventional treatments [169]. Many Gendicine-based clinical trials for the treatment of other types of cancers, such as ovarian, lung, breast, liver, pancreatic, and colorectal cancers, are ongoing [169].

Oncorine®, an AdV-based therapy for the treatment of late-stage refractory nasopharyngeal cancer, was approved in China in 2005 [170]. The recombinant virus selectively replicates in and lyses p53-deficient malignant cells as a result of viral E1B-55KD gene deletion [170]. Oncorine® is currently investigated in clinical trials for the treatment of other cancer types, such as refractory malignant ascites and hepatocellular carcinoma.

Rexin-G®, an RV-based gene therapy for the treatment of solid tumors, was approved in the Philippines in 2007. Expression of cyclin G1 in malignant cells leads to the G1 phase cell cycle arrest, apoptosis, and cell death. Several US-based phase I and II clinical trials have investigated this gene therapy approach for the treatment of breast, pancreatic, and other types of cancer [171–173]. It was approved for the treatment of osteosarcoma and soft tissue sarcoma in 2008 and for pancreatic cancer in 2009 [166].

Six AAV vector-based treatments, namely, **Glybera®** for the treatment of lipoprotein lipase deficiency, **Luxturna®** for retinal dystrophy, **Zolgensma®** for spinal muscular atrophy, **Upstaza®** for AADC, **Roctavian®** for hemophilia A, and **Hemgenix®** for hemophilia B, were approved in 2012, 2017, 2019, and 2022, respectively. The number of AAV-based phase I/II clinical trials has significantly increased. AAV vectors have demonstrated clinical efficacy in the treatment of several monogenic metabolic, ophthalmological, neurological, and hematological diseases [5, 174–176].

Three LV vector-based treatments, **Libmeldy®** for infantile metachromatic leukodystrophy, **Zynteglo®** for treatment of β -thalassemia, **Skysona®** for treatment of ALD, were also approved.

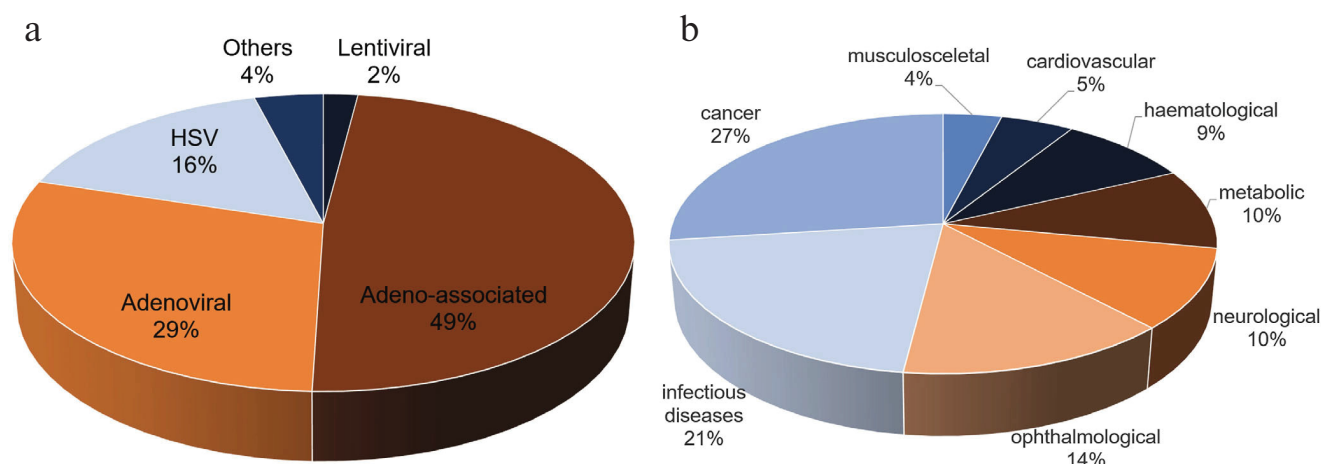


Fig. 7. Current clinical trials by viral vector (a) and therapeutic area (b) [1].

ONGOING CLINICAL TRIALS

Over 3000 gene therapy clinical trials based on viral vectors had been started, completed, and approved in the past thirty years. While ongoing clinical trials use more than ten vector types, they mainly rely on AAVs (49% of all clinical trials) and AdVs (29% of all trials) (Fig. 7a). Gene therapy approaches are tested mostly for the treatment of ophthalmological (14% of trials), neurological, metabolic (both 10% of trials), and hematological (9% of trials) disorders (Fig. 7b) [1].

Clinical trials for the treatment of monogenic disorders (mucopolysaccharidosis and Fanconi anemia) and various types of cancers (melanoma, hepatocellular carcinoma, head and neck, breast, lung, and prostate cancers) are ongoing [1]. An outstanding clinical potential of these therapies is reflected by the fact that many of them enter early-stage trials and many products have been proven safe and reached late-stage trials.

Approved **AdV vector**-based trials, both ongoing and completed, are focused mainly on infectious diseases and cancer. AdV vectors induce a strong pro-inflammatory response, which makes them highly potent tools for the treatment of cancer. The clinical trials mainly rely on AdV5 and AdV26 serotypes followed by EnAdV (chimeric serotype enadenotucirev), AdV VCN-01 and AdV5/35 (both human AdV5-based), and ChAdV (chimpanzee AdV), the latter becoming an important serotype due to its safety profile [177, 178]. The anti-cancer gene therapy candidates deliver genes encoding IFN- γ , IL-12, IL-15, 4-1BBL, and many others. Due to the high transgene expression and immunogenicity, these vectors are ideal candidates for the development of vaccines against hepatitis B, SARS-2 (severe acute respiratory virus-2), HIV, tuberculosis (TB), and other infectious pathogens [179]. In the past few years, the use of AdV vectors has declined, as AAV and LV vectors started to dominate the clinical trial landscape [180].

By mid-2023, the number of **AAV**-based trials reached 262 with more than 7% phase III trials approved, ongoing, or completed. Late-stage trials mainly focus on hematological diseases (hemophilia A and B), neurological diseases (spinal muscular atrophy), ophthalmological diseases (retinitis pigmentosa), and musculoskeletal disorders (DMD).

Historically, **RV vectors** had been used for the treatment of monogenic diseases, different types of cancer, and HIV. However, their application has declined due to the inability to transduce non-dividing cells and the risk of insertional mutagenesis [130]. The long-term safety of RV vectors has been assessed for fifteen years in 30 patients in Italy [181]. Only one RV-based therapy product, Strimvelis, was approved for the treatment of ADA-SCID as a result of hundreds of trials.

The number of **LV-based** approved, ongoing, or completed clinical trials has been steadily but surely increasing over the last ten years due to the ability of these vectors to transduce both dividing and non-dividing cells and low genotoxicity [182, 183]. A safer, modified SIN LV vector was used for the treatment of 65 HIV patients with no side effects for over 8 years post gene therapy [184]. While current active trials investigate gene therapy for a wide variety of diseases, they are predominantly focused on the treatment of monogenic diseases, infectious diseases, and cancer. The treatment of solid tumors is the focus of cancer clinical trials because of the ability of viral vectors to transduce solid tumors more efficiently. The landscape of monogenic disease trials includes ophthalmological, metabolic, neurological, hematological, musculoskeletal, and cardiovascular diseases [166].

CONCERNS: GENOTOXICITY AND SHEDDING

Both non-viral and viral factors can cause genotoxicity. The dose, nature of transgene, age of recipient, and disease state (immunodeficient patients are more

prone to dysregulation of proto-oncogenes) can influence genotoxicity, while the origin of a viral vector and its design, integration site, and type of targeted cells can cause genotoxicity as a result of proto-oncogene upregulation.

Inflammation, random integration affecting normal genes, upregulation of proto-oncogenes, and insertional mutagenesis can not only lead to the disruption or dysregulation of gene expression, but also cause clonal expansion and oncogenesis [185]. Adjacent proto-oncogenes can be activated by (i) **epigenetic gene silencing**, in which viral vector-induced gene methylation inhibits expression of neighboring genes due to histone and DNA methylation expansion; (ii) **promoter insertion**; and (iii) **gene transcript truncation** (loss-of-function mutations in tumor suppressor genes) [119, 186–188]. Different vectors display different cell-specific integration profiles. To overcome this hurdle, viral vectors with a lower risk of insertional mutagenesis (SIN vectors [186, 189]), enhancer-blocking chromatin insulators [190], and vector-targeting microRNA have been developed [191].

It is important to understand the effects of the vector on the immune system before proceeding to clinical trials, as acute immune response can lead to severe complications, and, in some instances, lethal outcomes [192]. Previous exposure to a viral serotype used in the therapeutic vector can result in a strong immune response due to pre-existing nAbs, thus decreasing the clinical efficacy and inflammation as a result of genotoxicity [192]. While up to 80% of the world population has been exposed to the AAV serotypes 1 and 2, other AAV serotypes are known to vary geographically. For example, AAV2 nAbs are prevalent in more than 50% of 407 samples from Africa compared to 30% of 100 samples from the United States, while AAV8 nAbs were prevalent in about 30% of samples from Africa compared to about 15% of samples from the United States [188–190].

And finally, virus shedding may be a cause of concern. Some studies showed that viral vectors can be excreted ('shed') from the blood, urine, saliva, and stool for up to a week after injection. General guidelines recommend caution between days 14 and 30 after vector administration for both health care professionals and family members [194–196].

CONCLUSION

More than 6000 rare diseases affect over 300 million people worldwide with devastating effects. Half of rare diseases affect children, 30% of whom will die before the age of 5 [197]. As few existing conventional therapies only manage the symptoms, new effective approaches, such as gene and cell therapies, have been long overdue. In contrast to conventional treatments, gene therapy drugs are mostly offered as a single-shot treatment, alleviating the burden of daily treatments.

Although not all inherited disorders can be treated with this unique approach, better results are usually achieved if the presymptomatic treatment has been used, and not all individuals respond uniformly, gene therapy offers a life-saving solution for patients with rare and often fatal disease. By now, effective and long-lasting gene therapy treatments have been developed for many disorders. As a result of research and development efforts, improved viral vectors are now able to evade pre-existing immunity, target and transduce therapeutically relevant cells more efficiently, ensure genomic maintenance, and express relevant levels of a transgene. Further focus on improving the efficacy and safety of viral vectors while reducing the necessary therapeutic dose, off-target effects, and production costs will ensure the success of gene therapy. It should be also mentioned that gene therapies often get accelerated approval, therefore, long-term follow-up is essential to ensure the durability of response or the long-term safety of newly approved therapies. Whereas hematological, neurological, and ophthalmological therapeutic areas have witnessed successful treatment of hemophilia and spinal muscular atrophy, restoration of the immune system in children with immunodeficiencies, and improvement of visual acuity, the majority of neurodegenerative and metabolic disorders are still "waiting in the wings". Undoubtedly, this brings hope to the patients with rare genetic disorders (and their families) of an improved quality of life and significantly longer life expectancy – something that so many healthy individuals take for granted.

Contributions. E.M. manuscript concept, writing and editing, preparation of figures; A.G. clinical data analysis and preparation of figures; A.E. manuscript writing, R.I. manuscript editing, A.K. manuscript concept and editing.

Funding. This study was supported by the Russian Science Foundation (project no. 22-14-20046). A.D.E. was supported by the Russian Science Foundation (project no. 22-14-20046). E.M., A.G., A.K., and R.A.I. were supported by the internal projects GTH-RND-2011 and GTH-RND-2112.

Ethics declarations. The authors declare no conflicts of interest. This article does not contain description of studies with the involvement of humans or animal subjects performed by any of the authors.

Electronic supplementary material. The online version contains supplementary material available at <https://doi.org/10.1134/S0006297923120179>.

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