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GENE THERAPY AND CLINICAL APPLICATIONS

Lamiel Membrilla, Alberto[1]

University of Barcelona, Faculty of Pharmacy and Food Science
Av. Joan XXIII, s/n 08028 Barcelona

Abstract

Nowadays, there are many diseases that are incurable or for which a complete remission treatment is not available. That is why gene therapy has emerged as a great alternative for these diseases. Gene therapy is a therapeutic strategy based on the genetic modification of cells, either *in vivo* or *ex vivo*, through the use of certain genetic editing methods such as programmable site-specific nucleases, like ZFNs, TALENs, CRISPR-Cas, etc., as the use of vectors responsible for transferring the gene to the target cell. Many clinical trials are underway and some have even been approved, resulting in the commercialization of gene therapy products to treat certain diseases.

Keywords: gene therapy, viral vectors, DNA delivery systems.

Resumen

Hoy en día, hay muchas enfermedades incurables o para las cuales no se dispone de un tratamiento de remisión completa. Por ello la terapia génica ha surgido como una gran alternativa a estas enfermedades. La terapia génica es una estrategia terapéutica que se basa en la modificación genética de las células, ya sea tanto *in vivo* como *ex vivo*, mediante la utilización de determinados métodos de edición genética como pueden ser las nucleasas programables ZFN, TALEN, CRISPR-Cas… así como el uso de vectores encargados de transferir el gen a la célula diana. Muchos ensayos clínicos están en proceso e incluso algunos han sido aprobados, dando como resultado la comercialización de productos de terapia génica para tratar ciertas enfermedades.

Palabras clave: terapia génica, vectores virales, sistemas de entrega de ADN.

Resum

Avui en dia hi ha moltes malalties incurables o per a les quals no es disposa d'un tractament de remissió completa. És per això que la teràpia gènica ha sorgit com una gran alternativa per a aquestes malalties. La teràpia gènica és una estratègia terapèutica que es basa en la modificació genètica de les cèl·lules, tant *in vivo* com *ex vivo*, mitjançant la utilització de determinats mètodes d'edició genètica com poden ser les nucleases programables ZFN, TALEN, CRISPR-cas..., així com l'ús de vectors encarregats de transferir el gen a la cèl·lula diana. Molts assajos clínics estan en curs i alguns fins i tot han estat aprovats i han donat com a resultat la comercialització de productes de teràpia gènica per tractar certes malalties.

Paraules clau: teràpia gènica, vectors virals, sistemes d'entrega d'ADN.

[1] Pharmacy Student (a.lamiel48@gmail.com)

1. Introduction

1.1. Gene therapy

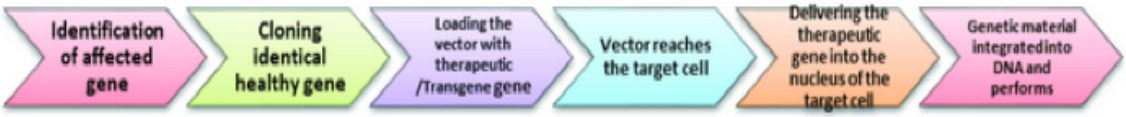
Gene therapy is defined by the National Institute of Health (NIH) as "an experimental technique that uses genes to treat or prevent diseases" (National Institute of Health, 2019). A more elaborate definition by Murali Ramamoorth and Aparna Narvekar is the next one: "Gene therapy is defined as the procedure used to treat or improve the health condition of the patient by modifying the patient's cells genetically. It provides a unique approach to treat both inherited and acquired diseases by delivering a therapeutic gene material and its associated regulatory elements into the nucleus; in order to correct the loss of function caused by mutation or to express the deficient gene product at physiologic levels" (Ramamoorth, 2016). Nowadays, researchers are testing several approaches for correcting faulty genes (Edge, 2005; Patil *et al.*, 2018):

- Replacing a non-functional or mutated gene with a healthy copy of the gene.
- Regulating a particular gene (*knock out/knock in*).
- Introducing a new gene and thus inducing the production of a therapeutic protein.
- Altering or killing an aberrant cell.

However, it is not a simple technique; quite the opposite and requires a long procedure.

First of all, it is essential to determine the physiopathology of the genetic disorder and consequently the involved gene. Secondly, it is necessary to target the proper cell in the injured tissue or organ. The most frequently used cells are skin fibroblasts, infiltrating lymphocytes and the liver as the main target organ (Rozalén, Ceña and Jordán, 2003). Finally, the selection of the right vector is a key point. After the vector is chosen, the gene of interest, or "therapeutic gene" or "transgene", is introduced into the vector which is able to do a transfection (Mele, 2012). Sequential steps in gene therapy are shown in Table 1.

TABLE 1: SCHEMATIC TABLE OF KEY STEPS IN GENE THERAPY.
Source: (Ramamoorth, 2016).



1.2. Types of gene therapy

We can distinguish two branches of gene therapy. The first one is when we refer to the nature of the targeted cell (somatic or germline). The second one is when we talk about where genetic manipulation takes place *(in vivo* or *ex vivo*).

On the one hand, if we talk of gene manipulation that results in a modification of the patient's germline and thus in their offspring, we are referring to germline gene therapy, which deals with germ cells (ovaries and sperm). By contrast, somatic gene therapy implicates introducing a gene into somatic cells, or non-germline cells, with the result of treating the patient without modification of their progeny. Currently, only somatic gene therapy is being practiced, and although germline therapy has the potential to prevent hereditary diseases, its application in humans raises many technical and ethical controversial issues (Rozalén, Ceña and Jordán, 2003; Mele, 2012; Patil *et al.*, 2018).

On the other hand, gene manipulation strategies can also be classified as: *in vivo* or *ex vivo* strategies. The *in vivo* approach is a direct strategy in which the vector is administered directly into the organ of interest or into blood vessels, and consequently genetic modification occurs in the host. In *ex vivo* gene therapy, frequently used in diseases where a specific type of cell is affected and can be easily obtained, such as hematopoietic stem cells (HSC), epidermal and limbal stem cells, cardiac stem cells, etc., the cell's genetic modification is done outside the body of the patient. After that, modified cells are reintroduced into the patient. *Ex vivo* approaches have two advantages: the first one is a stronger safety profile as a result of preventing direct human exposure to the vector that decreases its immunogenicity; the other is the possibility of selecting the target cells for transduction, thus improving specificity and efficacy (O'Connor and Crystal, 2006; Carvalho, Sepodes and Martins, 2017).

2. Material and Methods

Exhaustive bibliographical research on the subject of interest has been carried out.

A primary general search was done with *Google web search,* which was only used to have a comprehensive idea of the topic and to guide and structure the project. Once this consultation was concluded, databases specialized in biomedicine and other sciences, such as *PubMed* and *Scopus*, were used to carry out a more detailed search. In addition, some bibliography of the articles consulted was used to obtain more information.

Furthermore*,* webpages like *Genetics Home Reference* of the U.S. National Library of Medicine, which can be accessed at https://ghr.nlm.nih.gov/, have been consulted, as well as prestigious scientific journals such as *Nature* and *Science*.

3. Results

3.1. Methods for gene delivery

The choice of a proper vector is a key point in gene therapy, as the success of the therapy relies on ensuring that the therapeutic gene enters the target cell without any form of biodegradation. The ideal gene delivery system should have the following characteristics (Ibraheem, Elaissari and Fessi, 2014; Carvalho, Sepodes and Martins, 2017):

- High efficiency.
- Low toxicity without an immune response.
- Single cell specificity.
- Reproducibility and stability.
- Insertion of genetic material without size limit.
- Transduction in both dividing cells and non-dividing cells.
- Remain either in episomal position or integrate into a specific region of the genome, but not randomly.
- Easy to prepare and be inexpensive.

Several delivery techniques have been developed over the years. They are widely divided into two categories: viral vectors and non-viral vectors. Although non-viral techniques are rising, viral vectors remain by far the most used (Amer, 2014; Ginn *et al.*, 2018). The most common gene vectors used in clinical trials are adenoviral vectors, retroviral vectors, and naked plasmid, as shown in Figure 1.

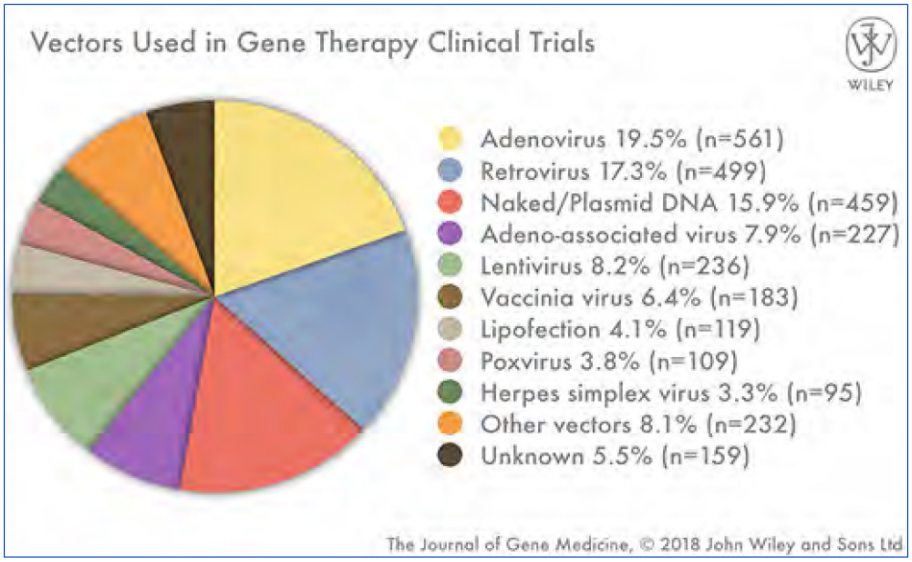


Figure 1: Graph of vectors used in gene therapy. Source: The Journal of Gene Medicine, Wiley and Sons (Edelstein, 2018).

3.1.1. Viral vectors

Viral vectors are viruses that have been genetically modified to carry a human gene. The pathogenic parts of its genes are removed, except those portions which are necessary to package the gene of interest and allow the virus to infect the cell. In other cases, like gutted adenoviral vectors, a helper DNA is used, as shown in Figure 2. This decreases the possibility of a host-immunogenic response and increases the safety profile of the vector, this being the major drawback of viral vectors (Ibraheem, Elaissari and Fessi, 2014; Benskey *et al.*, 2019). Other disadvantages are the limited size of a gene that a vector can carry, and that the production of viral vectors in huge quantities is very difficult and expensive. By contrast, viral vectors have high transduction efficacy, prolonged gene expression, and high cellular uptake (Ibraheem, Elaissari and Fessi, 2014; Carvalho, Sepodes and Martins, 2017). The principal viruses used as vectors are adenoviruses, adeno-associated viruses (AAV), retroviruses (lentivirus), and herpes simplex viruses (HSV). Each viral vector has both pros and cons, but there is no perfect vector for every gene therapy application (Benskey *et al.*, 2019).

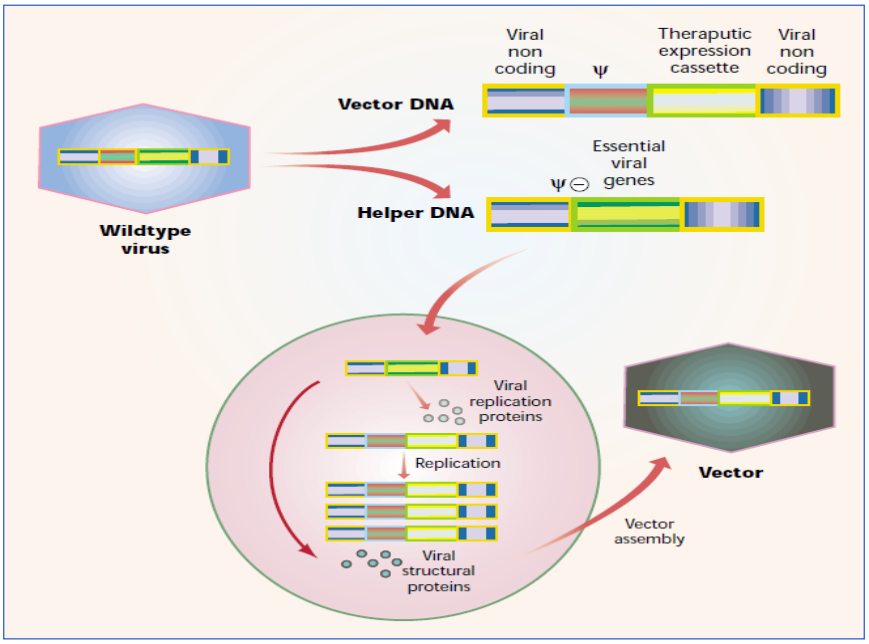


Figure 2: Generic strategy for engineering a virus into a vector. The helper DNA contains genes essential for viral replication and can be delivered as a plasmid, helper virus, or stably inserted into the host chromosomal DNA of the packaging cell. The helper DNA lacks the packaging domain (ψ) so it itself or its RNA cannot be packaged into a viral particle. The helper DNA of some vectors also lacks additional transfer functions, to increase safety. The vector DNA contains the therapeutic expression cassette and non-coding viral cis-acting elements that include a packaging domain. Some vectors contain viral genes that are relatively inactive (not transcriptionally active at the same level as in a wild-type infection) due to the absence of other viral genes. The viral proteins required for replication of the vector DNA are produced, leading to the synthesis of many copies of the vector genome (RNA or DNA, depending on the type of vector). Viral structural proteins recognize the vector (psi plus) but not the helper (psi negative) nucleic acid to result in packaging of the vector genome into a particle. Source: Kay, Glorioso and Naldini (2001).

Adenoviruses

Adenoviruses are naked double-stranded DNA viruses of approximately 30-35kb in length that cause respiratory (common cold), digestive, and eye infections in humans. They can infect both dividing and quiescent (non-dividing) cells. Their capacity of packaging is intermediate, less than 7.5kb of foreign DNA, providing short-term episomal expression in a relatively broad range of host cells.

Adenoviruses can infect a wide variety of cells through a specific interaction between the viral fiber protein and one cell surface receptor. The tropism of the virus can be altered by modifying the fiber protein so that it can bind more efficiently to other components of the cell surface. As mentioned, viruses have been modified (view Figure 3). In particular, adenoviruses need to be disarmed by crippling their replication system. This is done by deleting the E1 gene, important for viral gene expression and replication as it promotes transcription of other early viral genes, such as E2A, E3, and E4, and also binds to the host cell Rb protein, which prevents the cell from entering S phase. This prompts the host cell to express genes for DNA synthesis, which the virus utilizes for its own replication. The therapeutic gene replaces the E1 region. If the gene of interest is much longer, more than 5% longer than the wild type, packaging fails. To solve that, the E3 region, involved in blocking the im-

mune response to the virally infected cell, is deleted to keep the overall length of the DNA constant and secure good packaging.

One of the drawbacks is the high toxicity that may cause adverse effects due to a host immune response, like inflammation, as a consequence of repeated administration. Nevertheless, their immunogenicity makes adenoviruses perfect candidates to treat cancers and pandemic diseases; one case is *Gendicine*, the first adenovirus product approved to treat head and neck squamous cell carcinoma (Robbins and Ghivizzani, 1998; Kay, Glorioso and Naldini, 2001; Slade, 2001; Amer, 2014; Ibraheem, Elaissari and Fessi, 2014; Merten and Gaillet, 2016; Sridharan and Gogtay, 2016; Carvalho, Sepodes and Martins, 2017; Benskey *et al.*, 2019; Blejis, 2019).

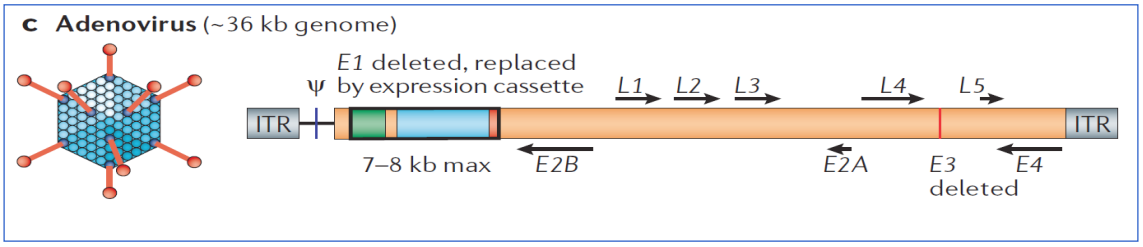


Figure 3: Adenovirus vector structure. *Expression cassette: Green: promoter/Orange: intron/Blue: transgene/Red: polyadenylation site. Source: O'Connor and Crystal (2006).

Retroviruses

A retrovirus is an enveloped virus that contains a reverse transcriptase enzyme that transcribes single RNA into double-stranded DNA when it enters the cytoplasm of the cell. As shown in Figure 4, all retroviral genomes have two long terminal repeat (LTR) sequences at their ends, needed for integration of the target genome into the host cell DNA. The LTR sequence frames the tandem *gag, pol* and *env* genes, encoding the structural proteins, viral protease (Pro)/reverse transcriptase (RT)/integrases (IN), and coat proteins, respectively. These genes are removed, allowing retroviruses to accommodate up to 8kb of foreign DNA, becoming completely defective in replication.

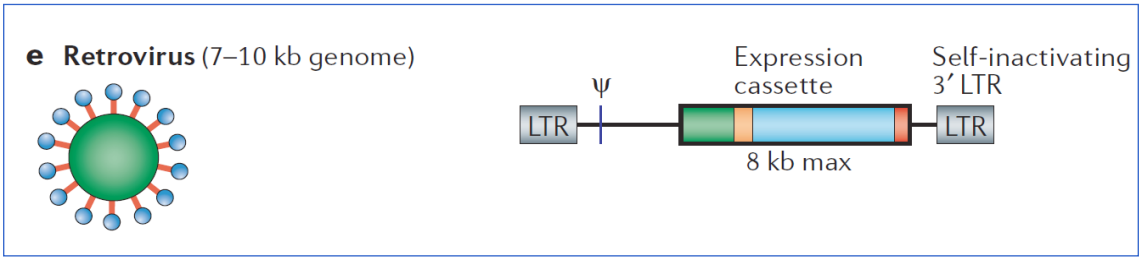


Figure 4: Retrovirus vector structure. Source: O'Connor and Crystal (2006).

Because of this, these genes must be provided by a packaging cell that lacks the packaging signal, so although it is responsible for the manufacture of the virus, it is not packed itself. Retroviruses infect a target cell through a specific interaction between the viral envelope protein and a cell surface receptor on the target cell. The virus is then internalized, where it is uncoated and the RNA reverse-transcribed into proviral dsDNA by means of the virally encoded *pol* gene. The dsDNA is then transported to the nucleus, where it is stably integrated into the host genome. That is the reason why retroviruses have represented the gold standard in vectors: for their long-term stable expression. However, they can just infect dividing cells, which is why lentiviruses, a genus of retrovirus based on

HIV, are being used instead, because lentiviruses can infect both dividing and non-dividing cells, possessing the same packaging capacity and chromosomal integration as conventional retroviruses (Robbins and Ghivizzani, 1998; Kay, Glorioso and Naldini, 2001; Slade, 2001; Amer, 2014; Ibraheem, Elaissari and Fessi, 2014; Merten and Gaillet, 2016; Sridharan and Gogtay, 2016; Carvalho, Sepodes and Martins, 2017; Benskey *et al.*, 2019; Blejis, 2019).

Adeno-associated viruses (AAV)

AAV are non-enveloped single-stranded DNA viruses that can infect both dividing and non-dividing cells and their tropism is very versatile, which makes this kind of virus very useful. Wild-type AAVs have the unique property of integrating into the human genome chr.19q13.3-qter. However, they need a helper virus, like adenovirus or herpes simplex virus, to infect a cell. AAV encode for two proteins, *rep* and *cap*, which are necessary for viral replication and integration. To generate an AAV vector *rep* and *cap* are deleted, and have to be supplied by an AAV helper virus. Only the virus terminal repeats are left (ITR) flanking the therapeutic gene as shown in Figure 5. They have a low capacity for packaging, less than 4.5kb, which is the major disadvantage. *Glybera* is one example of AAV commercialized nowadays used to prevent LPLD (Lipoprotein lipase deficiency) (Robbins and Ghivizzani, 1998; Kay, Glorioso and Naldini, 2001; Slade, 2001; Amer, 2014; Ibraheem, Elaissari and Fessi, 2014; Merten and Gaillet, 2016; Sridharan and Gogtay, 2016; Carvalho, Sepodes and Martins, 2017; Benskey *et al.*, 2019; Blejis, 2019).

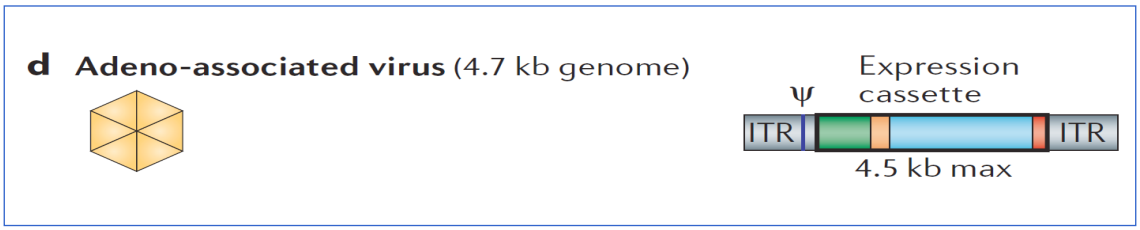


Figure 5: AAV vector structure. Source: O'Connor and Crystal (2006).

Herpes simplex viruses (HSV)

HSV are enveloped double-stranded DNA viruses and one of the main features is their tropism, being neurotropic (prefer nerve cells). To make HSV replication defective, a strategy similar to that used for adenovirus is employed. HSV have several proteins that are expressed early in infection, which activate expression from the other HSV promoters. Inactivating one or more of these immediate-early proteins (ICP0, ICP4, ICP22, ICP27) results in a vector unable to replicate, except with a helper. Another distinctive feature is their large capacity, about 152kb, making HSV the ideal vector for packaging and delivering large amounts of foreign DNA (up to 30kb), such as the pro-drug activating gene thymidine kinase enzyme, which enhances tumor lysis when ganciclovir is administrated in suicide gene therapy (Robbins and Ghivizzani, 1998; Kay, Glorioso and Naldini, 2001; Slade, 2001; Amer, 2014; Ibraheem, Elaissari and Fessi, 2014; Merten and Gaillet, 2016; Sridharan and Gogtay, 2016; Carvalho, Sepodes and Martins, 2017; Benskey *et al.*, 2019; Blejis, 2019).

TABLE 2: FEATURES OF PRINCIPAL VIRAL VECTORS USED IN GENE THERAPY.
Source: Del Hoyo Gil *et al.* (1999); Slade (2001); Amer (2014); Ibraheem, Elaissari and Fessi (2014); Carvalho, Sepodes and Martins (2017); Benskey *et al.* (2019).

Virus	Characteristics	Advantages	Disadvantages
Adenoviruses	Non-enveloped dsDNA Intermediate packed genome size (<7.5kb) In vivo	Very high titers High transduction efficiency Transduces many cell types Transduces proliferating and quiescent cells Remains episomal	Transient expression High immune toxicity
Adeno-associated viruses (AAV)	Non-enveloped ssDNA Low packed genome size (<4.5kb) Ex vivo	No pathogenic Low immunogenicity Remains episomal Prolonged expression Very versatile tropism High titers	Needs a helper virus Potential insertional mutagenesis Low transduction efficiency
Retroviruses	ssRNA (+) Intermediate packed genome size (8kb) Ex vivo In vivo (lentivirus)	Integration into cellular genome Prolonged stable expression Efficient transduction Low immunogenicity	Requires cell division for transduction (except lentivirus) Insertional mutagenesis Low titers
Herpes simplex viruses (HSV)	Enveloped dsDNA High packed genome size (>30kb) Ex vivo and In vivo	Prolonged expression Remains episomal Efficient transduction Neuronal tropism	Cytotoxic (high immunogenicity and pathogenic) Transient expression

3.1.2. Non-viral vectors

Non-viral vectors are promising (Sridharan and Gogtay, 2016). The drawbacks of viral vectors, such as immune response and limited gene size delivered, have prompted the development of this kind of vector (Ginn *et al.*, 2018). Non-viral vectors offer some advantages in relation to viral ones, like the ability to carry large gene size, more safety, low immunogenicity, an easy scale up production at low cost, and they can be stored for a long time due to their stability. Unfortunately, their high vulnerability to intra- and extracellular degradation, as well as low transfection efficiency, restricts the range of therapeutic applications. Non-viral delivery systems can be classified into three groups: physical, chemical, and bacterial (Baban *et al.*, 2010; Amer, 2014; Ibraheem, Elaissari and Fessi, 2014; Ramamoorth, 2016; Carvalho, Sepodes and Martins, 2017).

Physical

Physical techniques are based on the creation of transient pores in the cell membrane with the application of physical force, followed by the release of DNA by diffusion (Del Hoyo Gil *et al.*, 1999; Baban *et al.*, 2010; Amer, 2014; Ibraheem, Elaissari and Fessi, 2014; Ramamoorth, 2016).

- *Naked DNA*: the simplest technique where DNA is injected directly into the target tissue. The main advantages are the possibility to transfer large DNA, safeness, and simplicity. By contrast, DNA is quickly degraded and has a low transfection efficiency.

- *Electroporation*: inducing the uptake of injected DNA into the cell by increasing the permeability of the cell membrane through exposure to a controlled electric field. The main pros are safety, efficiency, and reproducibility. However, there are difficulties to transfer DNA into large areas of tissue and it requires surgery to install the electrode in the internal organs. In addition, it can cause harm and mutilation due to the high voltage applied.
- *Gene gun*: this method is also known as DNA-coated particle bombardment. It consists of delivering DNA using accelerated heavy metal (gold, tungsten or silver) particle carriers that are coated with the DNA. These particles should be small, biocompatible, and inert. The acceleration is provided either by water vaporization under a high-voltage electric spark or by using helium discharge. Some advantages are the high-level gene expression achieved quickly, the prolonged gene expression (compared to the other physical techniques), and the large range of cells that can be treated by this method.
- *Ultrasound*: increases the permeability of the cell membrane by using ultrasonic waves.

Chemical

In chemical techniques, DNA is carried by a chemical carrier which packs DNA either by electrostatic interaction between anionic DNA and polycations, or by encapsulating DNA with biodegradable polymers, or by adsorbing it (Del Hoyo Gil *et al.*, 1999; Amer, 2014; Ibraheem, Elaissari and Fessi, 2014; Ramamoorth, 2016).

- *Liposomes:* lipid vesicles capable of transporting several substances inside and, by fusion with the cell membrane (endocytosis), depositing the content inside the cells. Gene transfer can be performed either by transducing DNA inside the liposomes (anionic liposomes) or by forming complexes, since the polyanionic nucleic acids can stably bind with the micelle of the liposomes (cationic liposomes). Variants of cationic liposomes are Lipoplex or Polyplex (more stable). Liposomes can be administrated in different ways, such as injection, infusion, or even aerosol. Their advantages include selectivity to endothelial cells, good DNA protection, and low immunogenic response, but the main one is the ease of removal from the body due to polymer biodegradability.

Bacterial

Some bacteria, like Escherichia coli, Salmonella typhimurium, Clostridium, and Listeria, have the capability of delivering genetic material into cells (bactofection), especially tumour cells. Engineered bacteria are relatively safe, effective, and cheaper than viral vectors but, as a non-viral gene delivery system, the transfer of DNA into the nucleus is inefficient (Baban *et al.*, 2010; Amer, 2014).

3.2. Techniques of genome editing

New editing techniques have emerged in recent years. The most novel are site-specific nucleases, such as meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPR), and CRISPR-associated protein9 (CRISPR-CAS9), the newest one. These are the present and future of gene therapy (Giono, 2017). Their mechanism is similar in all cases, and is based on a double-stranded break (DSB) in specific DNA sites immediately corrected by the endogenous cellular repair machinery, either by homologous recombination (HR), also known as homology-directed repair (HDR), or by non-homolo-

gous recombination, also known as non-homologous end joining (NHEJ), and used to insert, delete, or correct a target gene permanently (Lee and Kumar, 2009; Giono, 2017).

Homologous recombination requires an exogenous sequence of DNA or just the homologous chromosome, used as a template, that will lead the repair making accurate changes in the target sequence. HR is used to make corrections or insertions of single or multiple transgenes (Gaj, Gersbach and Barbas, 2014; Maeder and Gersbach, 2016).

In contrast, NHEJ does not need a template DNA. Its mechanism is based on the direct merge of split ends. This repair pathway is prone to errors and often results in insertions or deletions (indels) at the site of the break; more frequently nucleotide removal occurs, interrupting the translational reading frame of the disrupted gene (frameshift), preventing or reducing expressions. This is why NHEJ is often used to knock-out a gene, but is not so good at repairing one (Carroll, 2017). In case two DSBs are formed simultaneously on the same chromosome, it is possible to delete a large segment of DNA between the two breakpoints, as shown in Figure 6 (Maeder and Gersbach, 2016).

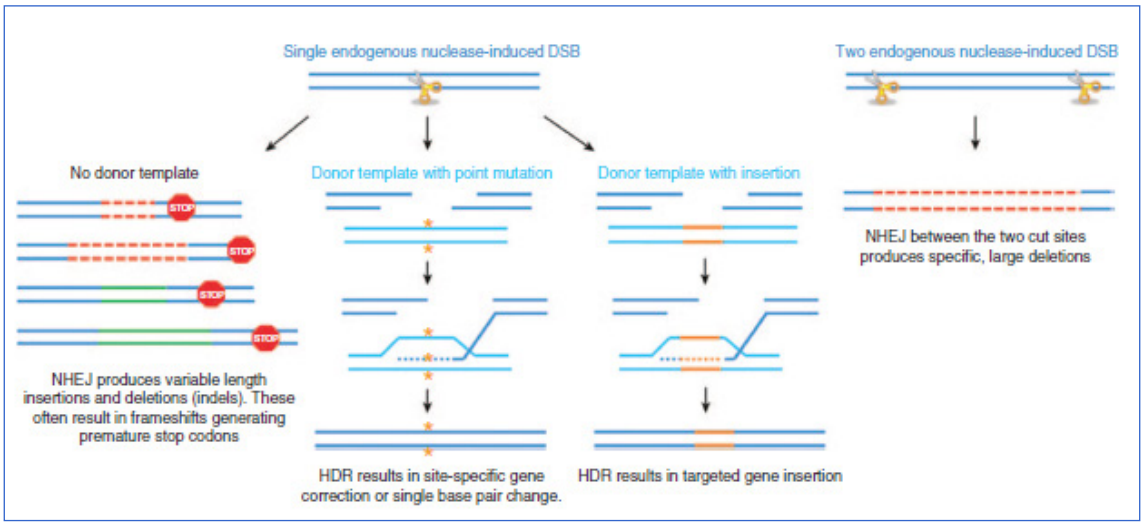


Figure 6: Mechanisms of double-stranded break repair. Source: Maeder and Gersbach (2016).

As mentioned before, there are four major programmable nucleases that can be classified into two categories depending on their mode of DNA recognition. On one hand, meganucleases, ZFNs, and TALENs detect DNA-binding domains via protein-DNA interaction. On the other hand, CRISPR-Cas9 detect DNA-binding domains through a short RNA guide that base-pairs directly with the target DNA (Cox, Platt and Zhang, 2015).

Meganucleases

Meganucleases, the smallest class of engineered nucleases (Maeder and Gersbach, 2016), are endonucleases with large recognition sites (14-40bp). Meganucleases have been used only for a short time due to deficiencies in specificity.

Moreover, re-targeting of meganucleases requires protein engineering (Cox, Platt and Zhang, 2015; Khadempar *et al.*, 2019).

ZFNs

Zin-finger nucleases are chimeric enzymes combining the zinc finger protein's DNA recognition specificity with the activity of the cleavage domain of the endonuclease FokI. Zinc-finger domains are usually constructed to contain three or more zinc fingers; each finger recognizes three base pairs (a triplet). When a ZFN is placed on both strands of the DNA, the FokI molecules form a dimer, activating the enzyme, and the midsection is cleaved creating a DSB (view Figure 7). ZFNs have also drawbacks, such as difficulty of the design, their re-targeting requires protein engineering, and inefficient multiple-gene targeting (Javed *et al.*, 2018).

TALENs

Transcription activator-like effector nucleases are chimeric enzymes consisting of a DNA binding domain (derived from TALE proteins) fused to the nuclease domain, FokI. Their mechanism is very similar to ZFNs (view Figure 7). One of the main differences between TALENs and ZFNs is that TAL effectors recognize individual nucleotides instead of triplets. Besides, re-targeting of TALENs requires complex molecular cloning (Javed *et al.*, 2018).

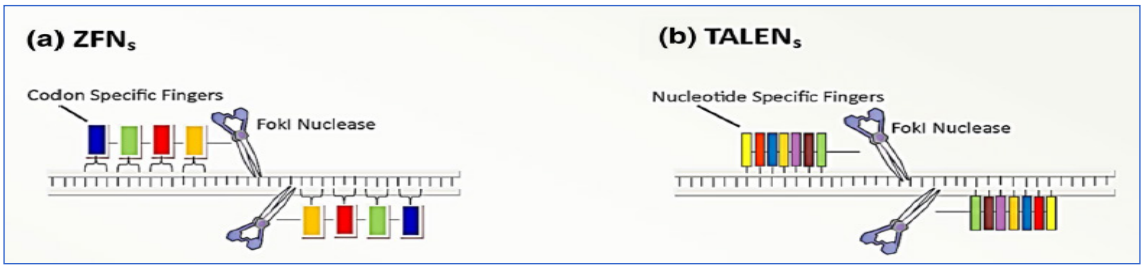


Figure 7: Mechanism of ZFNs and TALENs. Source: (Javed *et al.*, 2018)as the CRISPR-associated (Cas.

CRISPR-Cas9

Clustered regularly interspaced short palindromic repeats recently emerged; first published in 2012 (Lundin, Gissberg and Smith, 2015), as a potentially efficient alternative to ZFNs and TALENs for genome editing. It comes from the acquired immunity of bacteria and archaea used to combat viral infections. The mechanism is simple: a short sequence of foreign DNA is incorporated into CRISPR loci, between two repeated sequences known as a spacer, used as templates for the transcription into short RNA, known as crRNA (CRISPR RNA), and subsequently forms a complex (gRNA) with another RNA known as tracrRNA (trans-activating CRISPR RNA). The gRNA, or guide RNA complex, guides the Cas9 nuclease to recognize target DNA and induce a DSB. For this recognition it is also described that the Cas9 nuclease requires a PAM (protospacer adjacent motif): these are short nucleotide motifs that occur on crRNA (view Figure 8). The main advantages of CRISPR-Cas over the other techniques are simplicity and specificity. CRISPR-Cas protein is invariant and can be easily retargeted to new DNA sequences by re-designing the crRNA. Other advantages of this method are high efficiency and the production of multiple gene modifications simultaneously, as multiple gRNAs can be introduced at one time (Gaj, Gersbach and Barbas, 2014; Rath *et al.*, 2015; Cai *et al.*, 2016; Maeder and Gersbach, 2016; Giono, 2017; Javed *et al.*, 2018; Khadempar *et al.*, 2019). A CRISPR-Cas nuclease can also be used as an antimicrobial agent that can selectively destroy antibiotic-resistant strains, such as methicillin-resistant Staphylococcus aureus (MRSA) (Rath *et al.*, 2015; Javed *et al.*, 2018).

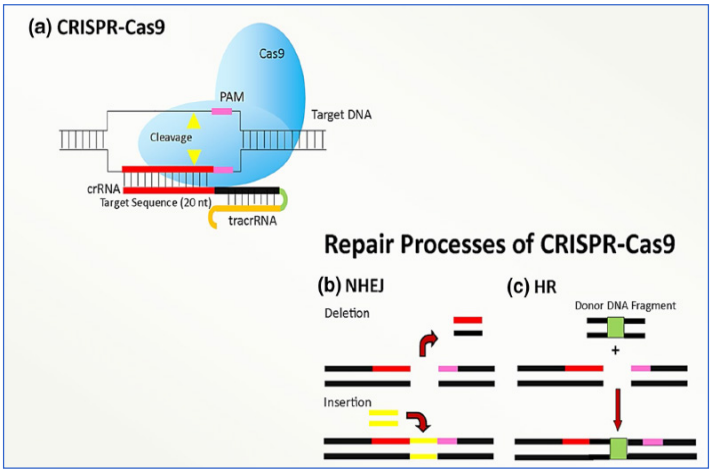


Figure 8: Mechanism of CRISPR-Cas9. Source: Javed *et al.* (2018).

Although these new genome-editing techniques offer many advantages in relation to the classic methods they still have limitations and complications, such as off-target effects, mosaicism, and multiple gene targeting difficulties, and that is why their use is still very restricted. However, every day these systems are trying to be improved in order to maximize efficiency, safety, and delivery (Gaj, Gersbach and Barbas, 2014; Maeder and Gersbach, 2016).

3.3. Clinical applications

Gene therapy is a promising therapeutic strategy which can be effective to cure a wide range of severe acquired and inherited diseases, such as hemophilia, acquired immunodeficiency syndrome (AIDS), cancer, and so on, based on using genes as a medicine (Ibraheem, Elaissari and Fessi, 2014). Nevertheless, it is not a new therapeutic idea. The first time gene therapy came to light was in 1947 thanks to Clyde Edgar Keeler. However, it just was a brief idea of what gene therapy could be (Keeler, 1947).

It was not until 1974 when the first direct human gene therapy trial took place. Two female patients suffering from hyperargininemia (urea cycle disorder) were treated with the wild-type Shope papillomavirus with the intention of introducing the gene for arginase. Unfortunately, the trial was unsuccessful due to no change in the arginine levels being perceived (Wirth, Parker and Ylä-Herttuala, 2013).

In 1988, Eve Nicols described the criteria for selecting those diseases that were candidates to be treated by gene therapy (Nichols, 1988):

- The disease is an incurable, life-threatening disease.
- Organ, tissue and cell types affected by the disease have been identified.
- The normal counterpart of the defective gene has been isolated and cloned.
- The normal gene can be introduced into the cells from the affected tissue.
- The gene can be expressed adequately and it will direct the production of enough normal protein to make a difference.
- Techniques are available to verify the safety of the procedure.

Sixteen years later in 1990, after the first direct human gene therapy trial, the Food and Drug Administration (FDA) approved the first human gene therapy assay. It was conducted by William French Anderson, also known as "the father of gene therapy", and his team. Autologous *ex vivo* modified white blood cells were transferred via recombinant retrovirus to two adenosine deaminase deficient (ADA) pediatric patients, also known as "the bubble boys' disease". Even though the trial demonstrated to be safe, its efficacy was not as expected. However, it was a big step in the world of gene

therapy. A little later, an ADA trial was done in Italy, and after that other tests were realized. But it was in 1999 when the first death occurred. Jesse Gelsinger, an 18-year-old boy suffering from a partial deficiency of ornithine transcarbamylase (OTC), took part in a gene therapy trial at the University of Pennsylvania, Philadelphia. Unluckily, his immune system responded immediately after a very high dose of adenovirus and that led him to death just a few days later as a result of multiorgan failure.

However, gene therapy did not stop developing and in 2003 China would be the first country to approve the first gene-therapy-based product for clinical use, called Gendicine™. It was approved for the treatment of head and neck squamous cell carcinoma, based on an adenoviral vector in which the E1 gene is replaced with a human p53 tumor-suppressor gene in order to stimulate apoptosis, increasing the expression of tumor suppressor genes and immune response factor cells. It also decreases the expression of multi-drug resistance, vascular endothelial growth factor, and matrix metalloproteinase-2 genes, and blocks transcriptional survival signals. Gendicine appears to act synergistically with conventional treatments such as chemo- and radiotherapy.

After two years, the Chinese State Food and Drug Administration (CSFDA) validated another gene therapy product, Oncorine™, for the treatment of late-stage refractory nasopharyngeal cancer. Oncorine is an oncolytic adenovirus to be used in combination with chemotherapy.

In 2004, Ark Therapeutics Group Plc received the first commercial GMP certification in the EU for the manufacture of Cerepro®, an adenoviral vector harboring the gene for the herpes simplex virus thymidine kinase (HSV-tk), used to treat brain tumors. In 2008 it completed phase III clinical trials, the first in history, but was never commercialized because the applicant never demonstrated to the CHMP (Committee for Medicinal Products for Human Use) clear evidence of a clinically significant benefit in relation to risk.

Finally, on 19 July 2012, the EMA (European Medicines Agency) recommended for the first time a gene therapy product, Glybera® (alipogen tiparvovec), for approval in the European Union. Glybera® is an adeno-associated viral (AAV) vector with an intact copy of the human lipoprotein lipase gene (LPL) for delivery to muscle cells, which was approved, after three attempts, for the treatment of severe lipoprotein lipase deficiency. Data from the clinical trials indicate that fat concentrations in blood were reduced between 3 and 12 weeks after injection, in nearly all patients. Glybera® opened the way for the authorization of several gene therapy products like Imlygic® (talimogene laherparepvec), an oncolytic viral therapy with life-attenuated HSV used to treat melanoma. In HSV, two genes are removed and one is added. The genes removed encode for the infected cell protein (ICP34.5 and ICP47) involved in blocking the response of healthy cells to stop replicating and suppressing an immune response to viral infection, respectively. A gene coding for granulocyte colony-stimulating factor (GM-CSF) is inserted to promote an immune response.

Another approved gene therapy product is Strimvelis®, the first ex vivo stem-cell gene therapy approved in Europe for patients with ADA-SCID who are not suitable for bone-marrow transplants because of a lack of a matching donor. The treatment is personalized for each patient; hematopoietic stem cells (HSCs) are extracted from the patient and purified so that only CD34-expressing cells remain. Those cells are cultured with cytokines and growth factors and then transduced with a gammaretrovirus containing the human adenosine deaminase gene and then reinfused into the patient. These cells take root in the person's bone marrow, replicating and creating cells that mature and create normally functioning adenosine deaminase protein, solving the problem. Around 75% of patients needed no further enzyme replacement therapy (Rozalén, Ceña and Jordán, 2003; Edge, 2005; Wirth, Parker and Ylä-Herttuala, 2013; Carvalho, Sepodes and Martins, 2017; Blejis, 2019).

More recently, in 2017 more products were approved by the FDA, such as Kymriah™ (tisagenlecleucel), the first CAR T-cell therapy product to treat acute lymphoblastic leukemia (ALL); Yescarta™ (axicabtagene ciloleucel), another CAR T-cell product for B-cell lymphoma, and Luxturna™ (vortigene neparvovec-rzyl), an AAV for retinal dystrophy in which the correct copy of the RPE65 gene is delivered and expressed in retina cells to halt the progression of the disease. Luxturna is injected directly into the retina (Ginn *et al.*, 2018; Blejis, 2019). A schematic summary is shown in Table 3.

TABLE 3: TIMELINE OF GENE THERAPY.
Source: author's own work.

1947	• Gene therapy comes to light
1974	• First direct human gene therapy trial (hyperarginemia)
1990	• First human gene therapy assay approved by FDA (ADA-SCID)
1999	• Jesse Gelsinger death
2003	• First gene therapy based product for clinical use (Gendicine™)
2005	• Oncorine™
2004-2008	• Cerepro®
2012	• First gene therapy product approved by EMA's recommendation (Glybera®)
2015	• Imlygic®
2016	• First ex vivo stem-cell gene therapy (Strimvelis®)
2017	• Kymriah™, Yescarta™, Luxturna™

To date, roughly 2600 approved gene therapy trials worldwide have been conducted or are still ongoing (Wirth, Parker and Ylä-Herttuala, 2013). As shown in Figure 9, cancer is the principal disease treated by gene therapy, followed by monogenic diseases (haemophilia, cystic fibrosis, Duchenne muscular dystrophy), infectious diseases (HIV, hepatitis B), and cardiovascular diseases.

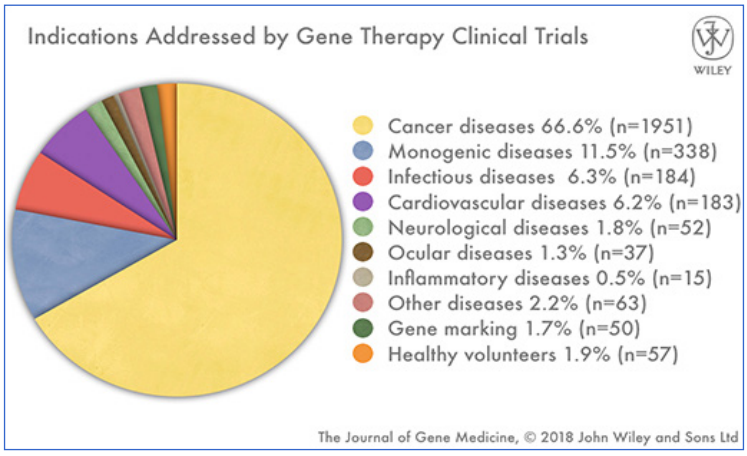


Figure 9: Graph of indications addressed by gene therapy in clinical trials.
Source: The Journal of Gene Medicine, Wiley and Sons (Edelstein, 2018).

Cystic fibrosis

A common severe autosomal recessive genetic disease which is caused by dysfunction of epithelial cell surface cAMP-activated chloride channels. Human morbidity results from the effects in the respiratory epithelium; loss of proper fluid transport in the lung results in thickening of the mucus and consequently infection and breathing complications (Maeder and Gersbach, 2016)the recent advent of genome-editing technologies has enabled a new paradigm in which the sequence of the human genome can be precisely manipulated to achieve a therapeutic effect. This includes the correction of mutations that cause disease, the addition of therapeutic genes to specific sites in the genome, and the removal of deleterious genes or genome sequences. This review presents the mechanisms of different genome-editing strategies and describes each of the common nuclease-based platforms, including zinc finger nucleases, transcription activator-like effector nucleases (TALENs. The gene re-

quired (CFTR) is well characterized and only low-level expression is needed. Adenovectors, carrying the gene encoding the functional CFTR protein, are the most advanced vectors, although liposomes have been used too (Patil *et al.*, 2018). Moreover, administration is very easy, achieved by using an inhaler (Rozalén, Ceña and Jordán, 2003; Carvalho, Sepodes and Martins, 2017).

HIV infection

The aims are to maintain the virus in its latent period for as long as possible and to protect uninfected cells from viral infection, and perhaps to enhance the immune response against the virus. To date, the most advanced gene-editing therapy is the *ex vivo* modification of T-cells to knock out C-C chemokine receptor type 5 (CCR5 – a coreceptor used for primary HIV infection). This study demonstrated a decrease in viral loads and an increase of CD4+ T-cells in HIV-infected mice (Cox, Platt and Zhang, 2015; Maeder and Gersbach, 2016). ZFN-mediated targeted integration of anti-HIV restriction factors into the CCR5 locus has led to the achievement of T-cells that offer almost complete protection from both R5 and X4-tropic strains of HIV (Gaj, Gersbach and Barbas, 2014). Modified T-lymphocytes are capable of recognizing cells infected by HIV and killing them (Rozalén, Ceña and Jordán, 2003). In addition, CRISPR-Cas9, via tail-vein injection, has been used in Khalili's lab to disrupt a crucial HIV gene (PSIP1) that encodes the LEDGF/p57 protein required for viral DNA integration into the host genome. The animals treated showed reduced expression of the HIV gene in multiple tissues, and thereby a reduction in viral infectivity (Cai *et al.*, 2016).

Cancer

Many different cancers are being targeted, including lung, gynecological, skin, neurological, gastrointestinal, pediatric tumors, and hematological malignancies, and several approaches to cancer therapy are being explored (Rozalén, Ceña and Jordán, 2003; Patil *et al.*, 2018), as follows.

1. Immunotherapy: immune responses to tumors are being enhanced, intensifying the normally weak humoral and/or cellular immune reactions to tumor antigens. Vaccination with tumor cells engineered to express immunostimulatory molecules, vaccination with recombinant viral vectors encoding tumor antigens, vaccination with host cells engineered to express tumor antigens, etc., are being employed as strategies (Ginn *et al.*, 2018). CAR T-cells (Chimeric Antigen Receptor T-cells) are highly effective at eradicating relapsed and refractory B-cell leukemias and lymphomas. Autologous CD8+ T-cells are engineered to recognize and kill cells bearing tumor-specific antigens, such as CD19 in B-cell malignancies, through a CAR that combines the specificity of a monoclonal antibody with the proliferative and cytotoxic abilities of an activated CD8+ T-cell (Amer, 2014; Kumar *et al.*, 2016). However, this method has limitations; for example, these modified T-cells express both the endogenous T-cell receptor as well as the engineered CAR, resulting in unpredictable epitope specificity and a reduction of potency. Another limitation is immune rejection, and that is why human leukocyte antigen (HLA) is knockout: to permit the immune system to discriminate between self and foreign cells (Maeder and Gersbach, 2016)the recent advent of genome-editing technologies has enabled a new paradigm in which the sequence of the human genome can be precisely manipulated to achieve a therapeutic effect. This includes the correction of mutations that cause disease, the addition of therapeutic genes to specific sites in the genome, and the removal of deleterious genes or genome sequences. This review presents the mechanisms of different genome-editing strategies and describes each of the common nuclease-based platforms, including zinc finger nucleases, transcription activator-like effector nucleases (TALENs. One example is Novartis' product Kymriah™, which has shown great promise in several clinical trials with complete remission (Carvalho, Sepodes and Martins, 2017)gene therapy medicinal products (GTMPs.

2. Oncolytic virotherapy: uses viruses capable of specifically targeting and replicating in tumor cells, causing cell lysis and killing the tumor. In some cases, these viruses are modified to encode immunostimulatory cytokines or tumor suppressor genes, such as p53, pRB, and BRCA-1, to enhance the antitumor immune response (Del Hoyo Gil *et al.*, 1999; Ginn *et al.*, 2018).
3. Gene-directed enzyme prodrug therapy, or suicide genes: genes are inserted into tumor cells to evoke cell suicide. These genes encode enzymes that convert prodrugs into cytotoxic drugs. For example, the herpes simplex virus thymidine kinase has been used to convert the nontoxic prodrug ganciclovir into the cytotoxic triphosphate ganciclovir. Another example is cytosine deaminase introduced by retroviruses or adenoviruses, which consists of the deamination of 5-flucytosine to fluorouracil, which inhibits cell division by blocking DNA synthesis and by forming RNA defective structures (Del Hoyo Gil *et al.*, 1999).
4. Cancer drug-resistance gene transfer: used in combination with radiotherapy and chemotherapy. Normal cells are modified with genes encoding drug resistance, especially to cytotoxic agents. For example, the MDR-1 gene (multidrug-resistant) in hematopoietic cells. With this procedure, higher doses of chemotherapy can be administrated, with lower toxicity and better efficiency (Del Hoyo Gil *et al.*, 1999; Amer, 2014).

As seen in Figure 9, more diseases than those mentioned above are treated with gene therapy; the following Table 4 shows some of these diseases and the applied gene therapy techniques.

TABLE 4: DISEASES TREATED BY GENE THERAPY AND THEIR THERAPEUTIC STRATEGIES.
Source: modified table from Rozalén, Ceña and Jordán (2003); Cox, Platt and Zhang (2015); Kumar *et al.* (2016); Maeder and Gersbach (2016); Jimenez *et al.* (2018); Patil *et al.* (2018); Kohn (2019); Stephens *et al.* (2019).

Disease	Gene Therapeutic Strategy
DMD (Duchenne muscular dystrophy)	NHEJ-mediated removal of a stop codon, and HDR-mediated gene correction.
HBV (hepatitis B virus)	NHEJ-mediated depletion of viral DNA inhibiting viral replication.
SCID (severe combined immunodeficiency)	HDR-mediated insertion of correct gene sequence.
Cataract	HDR-mediated correction of mutation in mouse zygote.
Hereditary tyrosinemia	HDR-mediated correction of mutation in liver.
Sickle cell disease and β-thalassemia	Correction of β-globin mutation in iPSCs and CD34+ HSCs, and inactivation of the enhancer of BCL11A.
Hypercholesterolemia	Gene disruption (PCSK9) in mouse liver to lower cholesterol.
α-1-antitrypsin deficiency	Gene correction in human iPSCs and differentiation into liver cells.
Epidermolysis bullosa	Gene correction in fibroblast and iPSCs.
Leber's congenital amaurosis	Deletion of an aberrant splice site.
Human papilloma virus	Inactivating essential viral genes.
Haemophilia	In vivo AAV vectors encoding cDNA factor VIII or IX.
Diabetes	Introduction of PDX-1 gene. Administration of a single injection of a therapeutic gene (FGF21) that decreases insulin resistance and decreases adipocyte size, without having side effects.

4. Discussion

Gene therapy is a promising therapy that has already been used successfully for certain diseases through the release of drugs approved by the regulatory administrators (FDA, EMA, CSFDA), such as Gendicine™, Oncorine™, Glybera®, Imlygic®, Strimvelis®, Kymriah™, Yescarta™, and Luxturna™, which until then were beyond the scope of previous therapies.

However, there is still a lot of development ahead in terms of safety, efficacy, ethics, and so on, which must be accomplished with strategic planning and rigorous process that allows regulatory processes to ensure the successful development of this class of medicines.

Numerous clinical trials are being carried out for this purpose, in addition to expanding the spectrum of diseases to be treated by gene therapy. It must also be said that genomic editing technology is advancing rapidly, which greatly favors gene therapy and is facilitating its development. Likewise, vectors, both viral and non-viral, one of the main problem, if not *the* main problem of gene therapy, are being studied and improved day-by-day with the aim of improving efficiency, specificity, safety, and delivery.

Nevertheless, these tools still need further refinement before they can be used safely and effectively in the clinic. Moreover, and without doubt, the ethical aspects regarding gene therapy need to be addressed. Some regulatory, economic, and socio-political issues must also be overcome before genetic medicines can become a reality, and working guidelines should be established.

Ultimately, we must keep in mind that transparency is the key for gene therapy to be accepted and that is why all information and knowledge available should be accessible to everyone.

5. Conclusion

After exhaustive bibliographic research on the topic of interest, several conclusions have been reached.

1. Gene therapy uses different technologies mainly based on the use of viral vectors, in particular, adenoviruses, retroviruses, and more recently adeno-associated viruses.
2. Non-viral vector technologies such as naked DNA and liposomes are improving day by day.
3. A novel field in gene therapy is gene editing using site-specific nucleases like ZFNs, TALENs, and CRISPR-Cas. These technologies are not yet available in the clinic, but may become new strategies in the future of gene therapy.
4. Nowadays, some diseases, especially monogenic diseases, have commercially available gene treatments. These diseases are: head and neck squamous cell carcinoma (*Gendicine*), refractory nasopharyngeal cancer (*Oncorine*), severe lipoprotein lipase deficiency (*Glybera*), melanoma (*Imlygic*), ADA-SCID (*Strimvelis*), acute lymphoblastic leukemia (*Kymriah*), B-cell lymphoma (Yescarta), and retinal dystrophy (*Luxturna*).
5. Gene therapy is not yet a standard therapy, but most of the scientific community believes, and claims, that gene therapy will be the future of the therapy. However, much improvement and research are required, as well as the establishment of ethical and legal bases.

6. Bibliography

Amer, M. H. (2014) "Gene therapy for cancer: present status and future perspective". *Molecular and Cellular Therapies*, 2(1), p. 27. doi: 10.1186/2052-8426-2-27.

Baban, C. K. *et al.* (2010) "Bacteria as vectors for gene therapy of cancer". *Bioengineered bugs*, 1(6), pp. 385–394. doi: 10.4161/bbug.1.6.13146.

Benskey, M. J. *et al.* (2019) *Viral Vectors for Gene Therapy: Methods and Protocols*, *Springer Nature*. Edited by F. P. Manfredsson and M. J. Benskey. New York: Springer (Methods in Molecular Biology). doi: 10.1007/978-1-4939-9065-8.

Blejis, D. A. (2019) *Gene Therapy Net*. Netherlands: Gene Therapy Net. Available at: http://www.genetherapynet.com/ (Accessed: 19 May 2019).

Cai, L. *et al.* (2016) "CRISPR-mediated genome editing and human diseases". *Genes and Diseases*. Elsevier Ltd, 3(4), pp. 244–251. doi: 10.1016/j.gendis.2016.07.003.

Carroll, D. (2017) "Genome Editing : Past, Present, and Future". *Yale Journal of Biology and Medicine*, 90, pp. 653–659.

Carvalho, M., Sepodes, B. and Martins, A. P. (2017) "Regulatory and Scientific Advancements in Gene Therapy: State-of-the-Art of Clinical Applications and of the Supporting European Regulatory Framework". *Frontiers in Medicine*. Frontiers, 4(182). doi: 10.3389/fmed.2017.00182.

Cox, D. B. T., Platt, R. J. and Zhang, F. (2015) "Therapeutic Genome Editing: Prospects and Challenges". *Nature Medicine*, 21(2), pp. 121–131. doi: 10.1038/nm.3793.Therapeutic.

Edelstein, M. (2018) *Gene Therapy Clinical Trials Worldwide*. UK: Abedia. Available at: http://www.abedia.com/wiley/indications.php (Accessed: 14 May 2019).

Edge, L. (2005) "The Future of Gene Therapy". *Biotechnology Healthcare*, (June).

Gaj, T., Gersbach, C. A. and Barbas, C. F. (2014) "ZFN, TALEN and CRISPR/Cas-based methods for genome engineering". *Trends Biotechnology*, 31(7), pp. 397–405. doi: 10.1016/j.tibtech.2013.04.004.ZFN.

Ginn, S. L. *et al.* (2018) "Gene therapy clinical trials worldwide to 2017: An update". *Journal of Gene Medicine*, 20(5), pp. 1–16. doi: 10.1002/jgm.3015.

Giono, L. E. (2017) "CRISPR/Cas9 y la terapia génica". *Medicina*, 77(5), pp. 405–409.

Del Hoyo Gil, L. *et al.* (1999) "Terapia Génica y biológica en el tratamiento del cáncer". *Farm Hosp*, 23(3), pp. 158–169.

Ibraheem, D., Elaissari, A. and Fessi, H. (2014) "Gene therapy and DNA delivery systems". *International Journal of Pharmaceutics*. Elsevier B.V., 459(1–2), pp. 70–83. doi: 10.1016/j.ijpharm.2013.11.041.

Javed, M. R. *et al.* (2018) "CRISPR-Cas System: History and Prospects as a Genome Editing Tool in Microorganisms". *Current Microbiology*. Springer US, 75(12), pp. 1675–1683. doi: 10.1007/s00284-018-1547-4.

Jimenez, V. *et al.* (2018) "FGF21 gene therapy as treatment for obesity and insulin resistance". *EMBO Molecular Medicine*, 10(8), p. e8791. doi: 10.15252/emmm.201708791.

Kay, M. A., Glorioso, J. C. and Naldini, L. (2001) "Viral vectors for gene therapy: The art of turning infectious agents into vehicles of therapeutics". *Nature Medicine*, pp. 33–40. doi: 10.1038/83324.

Keeler, C. E. (1947) "Gene Therapy". *J.Hered*, 38(10), pp. 294–298.

Khadempar, S. *et al.* (2019) '"CRISPR–Cas9 in genome editing: Its function and medical applications". *Journal of Cellular Physiology*, 234(5), pp. 5751–5761. doi: 10.1002/jcp.27476.

Kohn, D. B. (2019) "Gene therapy for blood diseases". *Current Opinion in Biotechnology*. Elsevier Ltd, 60, pp. 39–45. doi: 10.1016/j.copbio.2018.11.016.

Kumar, S. R. *et al.* (2016) "Clinical development of gene therapy: results and lessons from recent successes". *Molecular Therapy - Methods and Clinical Development*. Official journal of the American Society of Gene & Cell Therapy, 3(December 2015), p. 16034. doi: 10.1038/mtm.2016.34.

Lee, S. K. and Kumar, P. (2009) "Conditional RNAi: Towards a silent gene therapy". *Advanced Drug Delivery Reviews*. Elsevier B.V., 61(7–8), pp. 650–664. doi: 10.1016/j.addr.2009.03.016.

Lundin, K. E., Gissberg, O. and Smith, C. I. E. (2015) "Oligonucleotide Therapies: The Past and the Present". *Human Gene Therapy*, 26(8), pp. 475–485. doi: 10.1089/hum.2015.070.

Maeder, M. L. and Gersbach, C. A. (2016) "Genome-editing technologies for gene and cell therapy". *Molecular Therapy*. Official journal of the American Society of Gene & Cell Therapy, 24(3), pp. 430–446. doi: 10.1038/mt.2016.10.

Mele, C. (2012) "Gene Therapy: A Genetic Era of Technological Development to Treat Pediatric Genetic Disorders". *Journal of Pediatric Nursing*. Elsevier B.V., 27(2), pp. 180–183. doi: 10.1016/j.pedn.2011.12.004.

Merten, O. W. and Gaillet, B. (2016) "Viral vectors for gene therapy and gene modification approaches". *Biochemical Engineering Journal*. Elsevier B.V., 108, pp. 98–115. doi: 10.1016/j.bej.2015.09.005.

National Institutes of Health (2019) *What is gene therapy?*, *Genetics Home Reference*. Rockville Pike, MD: U.S. National Library of Medicine. Available at: https://ghr.nlm.nih.gov/primer/therapy/genetherapy (Accessed: 20 February 2019).

Nichols, E. K. (1988) *Human Gene Therapy*. Cambridge, MA: Harvard University Press.

O'Connor, T. P. and Crystal, R. G. (2006) "Genetic medicines: Treatment strategies for hereditary disorders". *Nature Reviews Genetics*, 7(4), pp. 261–276. doi: 10.1038/nrg1829.

Patil, S. R. *et al.* (2018) "Gene therapy: A comprehensive review". *International Medical Journal*, 25(6), pp. 361–364.

Ramamoorth, M. (2016) "Non Viral Vectors in Gene Therapy- An Overview". *Journal of Clinical and Diagnostic Research*, 9(November 2013), pp. 1–6. doi: 10.7860/jcdr/2015/10443.5394.

Rath, D. *et al.* (2015) "The CRISPR-Cas immune system: Biology, mechanisms and applications". *Biochimie*. Elsevier B.V, 117, pp. 119–128. doi: 10.1016/j.biochi.2015.03.025.

Robbins, P. D. and Ghivizzani, S. C. (1998) "Viral vectors for gene therapy". *Pharmacol. Ther*, 80(1), pp. 35–47.

Rozalén, J., Ceña, V. and Jordán, J. (2003) "Aplicaciones de la terapia génica". *Offarm*, 22(10), pp. 142–150.

Slade, N. (2001) "Viral vectors in gene therapy". *Periodicum Biologorum*, pp. 139–143. doi: 10.3390/diseases6020042.

Sridharan, K. and Gogtay, N. J. (2016) "Therapeutic nucleic acids: current clinical status". *British Journal of Clinical Pharmacology*, 82, pp. 659–672. doi: 10.1111/bcp.12987.

Stephens, C. J. *et al.* (2019) "Long-term correction of hemophilia B using adenoviral delivery of CRISPR/Cas9". *Journal of Controlled Release* (Elsevier), 298 (November 2018), pp. 128–141. doi: 10.1016/j.jconrel.2019.02.009.

Wirth, T., Parker, N. and Ylä-Herttuala, S. (2013) "History of gene therapy". *Gene*. (Elsevier B.V.), 525(2), pp. 162–169. doi: 10.1016/j.gene.2013.03.137.