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Avanços Recentes em Terapia Génica

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Aos meus pais,

que me permitiram sonhar.

Aos meus avós,

que sempre me motivaram.

Resumo: A terapia génica é um campo de estudo vasto e complexo que tem evoluído rapidamente nos últimos anos. Novas técnicas surgiram e outras foram aprimoradas, possibilitando a realização de ensaios clínicos que resultaram na aprovação de terapias génicas por respeitadas agências reguladoras, nomeadamente FDA e EMA. Desta forma, é indispensável esclarecer a comunidade médica sobre as técnicas em que assentam os progressos desta área de estudo. Neste trabalho, procura-se inicialmente clarificar os principais conceitos teóricos sobre as técnicas mais recentes de terapia génica, com enfase na utilização de vectores virais e sistemas CRISPR/Cas9. Considerando a terapia génica como a Medicina do futuro, o principal objectivo deste trabalho é perceber quais são as técnicas de edição genética mais utilizadas em ensaios clínicos actualmente em curso, bem como reconhecer os avanços alcançados, baseando a discussão numa revisão sistemática. Após a análise dos dados obtidos, pretende-se discutir de forma crítica sobre os obstáculos a transpor de modo a alcançar a manipulação assertiva e eficaz do genoma humano, bem como as implicações de descobertas recentes para a Medicina actual. O que trarão as terapias génicas ao futuro da Medicina?

Palavras-chave: terapia génica; vectores virais; CRISPR/Cas; AAVs; ensaios clínicos;

Abstract: Gene therapy is a rapidly evolving, vast, and complex field of study. In recent years, new techniques emerged and others were improved, allowing the design of gene therapy based clinical trials, that led already to the approval of some treatments for rare diseases by respected regulatory agencies, such as FDA and EMA. Clarifying the medical community about the techniques on which this progress was built is indispensable. In this work, I start by describing the theorical concepts behind gene therapy techniques, with an emphasis on viral vectors and CRISPR/Cas systems. Considering gene therapy as the future of Medicine, the main goal of this review is to understand which editing techniques are mostly used on clinical trials nowadays, as well as to recognize their results. A search was conducted to gather the most recent clinical trials using gene therapies. The analysis of results is expected to lead to a discussion about the barriers left to overcome to achieve an effective manipulation of the human genome, as well as the implications of recent discoveries to current medical practice. How is gene therapy shaping the future of Medicine?

Key words: gene therapy; viral vectors; CRISPR/Cas; AAVs; clinical trials;

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1. THEORETICAL CONCEPTS

1.1. Historical Perspective

The concept of gene therapy was established nearly 50 years ago when scientists hypothesized that genetic diseases, especially monogenic diseases, should benefit from the development of techniques that could replace the damaged gene. At that time, genetic diseases were mainly treated with gene product replacement, apart from diet and drugs that blocked or reduced the accumulation of undesired metabolites. Opposing to enzyme replacement therapies, gene therapy offered the possibility of achieving long-term and potentially curative responses, with no more than one treatment.

In the early 1990's, applications using gene therapy products in humans began.[1] It was not until some years later that effective results were achieved on treating SCID (Severe Combined Immunodeficiency) patients which represented a breakthrough in modern medical sciences.[2,3] A few accomplishments followed, regarding treatment of other conditions, such as epidermolysis bullosa[4] and Leber's amaurosis[5,6].[7] Unfortunately, several serious adverse events occurred in some patients, including metabolic and immune-mediated reactions, as well as the appearance of leukemia in treated SCID patients, which was related to the inadvertently activation of proto-oncogenes, and brought the attention back to the unknown and potentially dangerous risks of editing techniques used at the time.[8–10]

Over time, the growing knowledge at a molecular level led to the development of safer techniques and expanded therapeutic effects. Currently, there are several gene therapies approved by the Food and Drug Administration (FDA), reviewed ahead. Gene editing and delivery techniques continue under investigation and development. Figure 1 shows a timeline perspective of milestones achieved in clinical applications and investigation of editing and delivery techniques.

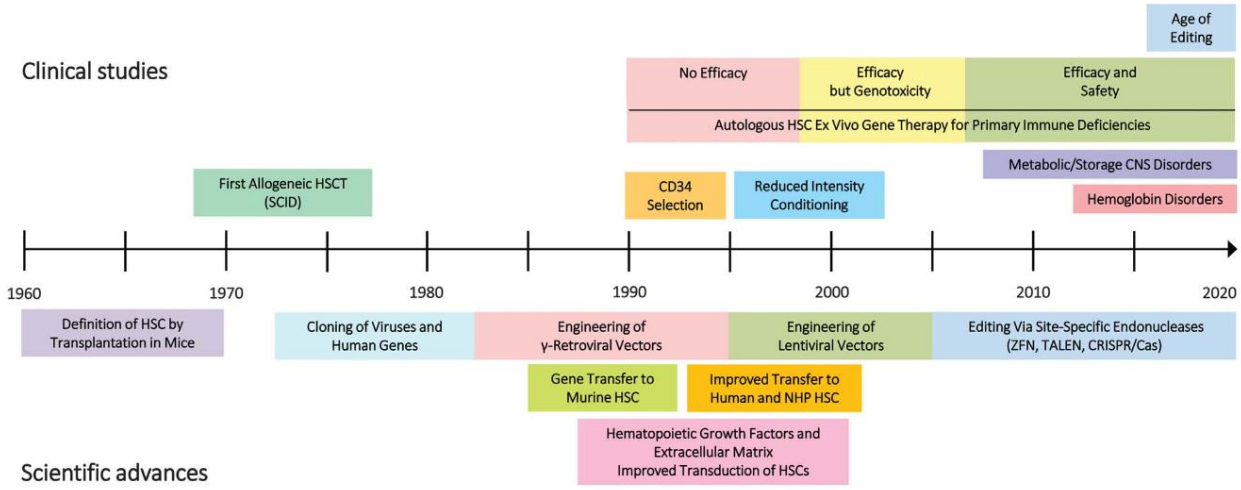


Figure 1: A timeline of advances in the field of gene therapy, from *Gene therapy comes of age*, by Dunbar et al.[11], marking the most important advances achieved thought years of investigation until 2020, comparing the timing of scientific breakthroughs to clinical applications.

1.2. Gene Therapy Definition

Gene therapy is defined by the FDA as modification or manipulation of gene expression or as the alteration of the biological properties of living cells for therapeutic use by either i) replacing a disease-causing gene with a healthy copy of the gene, ii) inactivating a disease-causing gene that is not functioning properly or iii) introducing a new or modified gene into the body to help treat a disease.

However, manipulation of gene expression does not imply a genome editing process and living cells do not refer specifically to human host cells, which leads to a broad definition that includes many cellular and immunotherapy treatments that use gene therapy techniques without actually editing the human genome.

In the context of this thesis, I will focus on gene modification techniques that target disease-causing genes. Procedures not involving human host genome manipulation,

such as *gene expression regulation*, interfering with mRNA translation, as well as methods designed to promote immune responses, will not be addressed. Some examples of technologies that will not be covered in depth in this review are RNAi, ASOs, vaccines and CAR T-cells.

In order to limit the previous definition to the framework of this review, gene therapy will be considered as any procedure that involves the manipulation of a patient's autologous cells' genome, by i) removing or replacing a defective genetic sequence known to cause a disease, or by ii) inserting a new gene with therapeutic purposes, in vivo or ex vivo, through chromosomic or episomal genome integration.

1.3. Editing Technologies

Genome editing is the controlled and deliberate change of the genome through insertion, deletion, replacement or correction of genes, and comprises three main procedures:

- Gene knockout: consists of disrupting a specific gene leading to loss of function;
- Gene correction: involves modifications in a specific genetic sequence to correct a disease-causing mutation;
- Gene addition: defined as the insertion of a functional copy of an existing defective gene (replacement) or as the insertion of a new gene with a therapeutic purpose, not necessarily due to loss of function mutations.

Any of the previous techniques can be executed *in vivo*, implying direct administration on the human body, systemically or to a specific location using an adequate delivery method, or *ex vivo*, which involves the removal, manipulation in culture, and reintroduction into the body of specific cells.

Several attempts at successfully shaping a disease natural course by genomic manipulation have been made. Before the modern era of engineered genetic manipulation tools, specialized enzymes such as integrases, recombinases, transposases

and endonucleases have been studied for site-specific genome modifications. However, the available variety of naturally occurring enzyme-specific sites was not sufficient to suit human DNA complexity, and the length, base pair composition of the target sequence was not enough to avoid off-target effects. The challenge became to redesign the domains responsible for DNA binding in these enzymes, to suit specific target sequences. The use of integrases, recombinases and transposases fell behind as the integration-site domains were not specific enough, and endonucleases arose as the most site-specific naturally occurring proteins, with the least off-site integrative events.[7] Other potential tools for genome editing such as transposons, recombinases and chemical cutters will not be approached in this review, due to their low relevance in clinical applications.

Researchers invested on the use of restriction enzymes, a type of endonucleases that cause site-specific double-strand breaks on DNA, and depend mainly on cell machinery to make the repair.[7] The main goal of scientific investigations in this area became the engineering of site-specific endonucleases to target any desired sequence with enough length to limit off-site activity and produce a double strand break in the DNA.[7]

It is important to understand that the initial goal was to cause a directed damage to the DNA at specific sites, although there was no control over what would succeed after that. Most of the times, the cell would repair the damage via Non-Homologous End Joining (NHEJ) and the loss of nucleotides would, hopefully, cause gene silencing.[7] The evolution in this field produced increasingly effective approaches and a better understanding of cellular mechanisms. More recent developments in genome editing techniques, particularly TALE nucleases and CRISPR/Cas systems, aim to use cellular DNA repair pathways, especially the Homology Directed Repair (HDR) pathway, to achieve precise genetic modifications, including precise integration of new genetic material, correction of mutated sequences or gene silencing, according to the repair pathway and the scientific approaches used.

Meganucleases, zinc-finger nucleases, and transcription activator-like effectors nucleases emerged as the first nuclease-based editing technologies developed to fulfil this goal and will be discussed ahead. These editing techniques emerged in the 80's, 90's

and 2000's, respectively, and evolved side by side with therapeutic gene delivery by viral vectors. However, their engineering and production are very intricate, expensive and time consuming, with fluctuating effectiveness.[12] The revolution in the field of gene editing platforms decisively arrived around 2012, when clustered regularly interspaced short palindromic repeat (CRISPR) in combination with CRISP-associated protein (Cas) were identified as an adaptive immune system in prokaryotes that could be used as a new genome editing platform[13], representing a faster and cheaper method than the previously used. Although it is not perfect, this system has been under extensive research and several advances have been already achieved since its discovery. CRISP-Cas based genome editing system represents the front-line technology in this area of knowledge and will receive particular attention in this review.

1.3.1. Meganucleases

Meganucleases emerged in the 80's and were the first endonucleases used in gene therapy. They are a type of restriction enzymes, capable of recognizing a DNA sequence up to 14-40 bp long, enclose high specificity to target sequences and are present in numerous organisms, and hundreds of naturally occurring meganucleases have been identified.[7]

Meganucleases work as "genome scissors", recognizing specific genomic sequences and producing a double-strand break at these sites. Then, the cell's genome repair machinery comes into action, and meganuclease-driven double-strand breaks frequently promote homologous repair, creating a window for targeted gene insertion or correction, if homologous sequences are added to the mix, called the "repair matrix", enabling gene insertion or correction.[14] In the absence of a repair matrix, the cell will most likely resort to NHEJ, and both ends of the double-strand break are reconnected based on micro-homology between the spared sequences, potentially causing deletions or insertions of diverse sizes. Hundreds of base pairs can be lost, resulting in deletions that frequently cause gene silencing. Also, if the endonuclease cleaves the gene between two direct repeats, the sequence contained in between can be deleted and the

ends joined by tandem repeat recombination. Additionally, if the cleavage occurs in two different sites, separate by some kb, large deletions can be achieved.[7]

However, the chances of finding a restriction enzyme that specifically recognizes the target DNA sequence are very slim, due to the multiplicity of possible combinations in human DNA. Meganucleases are composed of a DNA binding domain, constituted of 2 subdomains, and a catalytic center. The minor modularity of the DNA-binding domain poses as an obstacle to the adaptive engineering of the genomic interface.[7] Over time, other techniques emerged and today meganucleases are outdated as a potential tool for gene therapies, mainly because they are too difficult to engineer to target specific sites, compared to other recent techniques. Although safer and more effective technologies have become available, explaining where the idea of targeted genome editing approaches arose, through introducing double-strand breaks, seemed important to introduce the next editing technologies.

Essentially, all these systems rely on cellular machinery to double-strand break (DSB) repair. Eukaryotic cells can repair DSBs made to the DNA through NHEJ or HDR. Therefore, artificially inducing DSBs at strategic sites could lead to DNA repair by cellular mechanisms. Then, the cell DNA repairing machinery would finish the process following either the NHEJ or HDR pathways. The NHEJ pathway is error-prone and frequently causes insertions or deletions (indels) that lead to frameshifts or to a premature stop codon, often silencing the gene or originating a defective and useless protein. This pathway is the most frequently used by eukaryotic cells, since HDR needs certain conditions to occur. However, is through HDR that specific modifications can be made with higher precision. In this case, the use of this pathway depends on the availability of a homologous template sequence. Trying to control the pathway chosen by the cell is thus a crucial step to achieve precise DNA modifications. Increasing the HDR pathway activity depends on chemically inhibiting NHEJ pathway, the one most frequently used by the cell, and providing a repair template (double-stranded DNA plasmid or single-stranded DNA) that must be available at the time the DSB is made.

1.3.2. Zinc Finger Nucleases

Zinc finger nucleases (ZFNs) are a type of artificial restriction enzymes consisting of an engineered construct of DNA-binding zinc finger protein domain and a nuclease domain. The nuclease domain was developed using FokL, a bacterial nuclease which has modular structure that allows the physical separation between the nuclease domain and its DNA binding domain, which can be replaced with a module of DNA-binding zinc fingers.[13]

Zinc fingers (ZFs) are naturally occurring proteins that bind to DNA. A structure of three ZFs binds to DNA in a modular way, with each finger binding to a specific three nucleotide combination.[13] This qualified ZFs as a potential tool to target specific sequences, by combining different fingers known to recognize specific nucleotide triplets. Further engineering techniques were able to develop ZF modules, built by three individual fingers that recognized specific base triplets, connected to the nuclease domain of FokL.[13] This nuclease domain must dimerize to cut the DNA, and although wild-type FokL nucleases can cut DNA by forming homodimers, nucleases used in ZFNs were engineered to only execute cuts in heterodimeric forms.[7] By delivering two paired constructs, where each FokL monomer is linked to a different ZF module that recognizes specific sequences, if two constructs bind to complementary DNA strands, in opposite directions with inverted orientation, at close range, the corresponding monomers of FokL form a functional heterodimeric nuclease and make a double strand break in the spacer sequence.[15] The sequence between the two ZF DNA-binding modules is called spacer sequence. The site-specificity of the cut is increased by this dual-system.[15] An illustrative scheme is represented in figure 2.

This system has already been used in clinical trials regarding HIV (NCT00842634, NCT02500849) and hemophilia (NCT02695160), for instance. It was employed *ex vivo* in autologous CD34+ cells and *in vivo* using electroporation and AAV vectors; in this last approach, the vectors also delivered a template sequence to be used by the HDR pathway. However, engineering such constructs is expensive and intricate, obstructing a wider use of this technique to produce gene therapies.

1.3.3. TALE Nucleases

Transcription activator-like effectors (TALEs) are DNA binding proteins used by some plant infecting bacteria to highjack the cellular machinery and promote infectious capacity, by targeting the nucleus and binding to promoter regions, manipulating gene expression. TALE DNA-binding domains are constituted of tandem repeats, each 33 to 35 amino acids long, and positions 12 and 13 confer the nucleotide specificity, hence their naming as repeat-variable di-residues (RVD).[16] Domain modularity and the pairing predictability between each domain and a specific nucleotide, allowed the use of engineering strategies to build custom sequences of protein domains.

TALENs, transcription activator-like effectors nucleases, are engineered constructs containing TALEs and a FokL nuclease, similarly to what has been described for ZFNs. The cleavage process is like the one described for ZFNs, depending on the formation of an assembled and functioning FokL nuclease within an adequately sized spacer sequence of 10 to 30 bp, through the merging of the two paired TALEN constructs.[15] Due to the longer size of these proteins, concerning their DNA-binding domains, the specificity is greatly increased. The fact that there is at least one protein domain to bind to each nucleotide led to the development of a complementarity code, which assisted the construction process of TALENs designed for specific sequences, targeting a 30-40 bp of DNA sequence.[15]

Although their specificity is superior to ZFNs, due to a larger sequence recognition region, while displaying inferior cytotoxicity, their size complicates the delivery process and off-target mutagenesis still occurs with TALENs, although rational design can help reduce the probability of targeting similar sequences, this is an intricate process.[16]

Nonetheless, this editing platform has already been used in clinical trials regarding manipulation of CAR-T cells to target leukemia and multiple myeloma (NCT04150497, NCT04142619) and HPV-related cervical cancer (NCT03057912, NCT03226470).

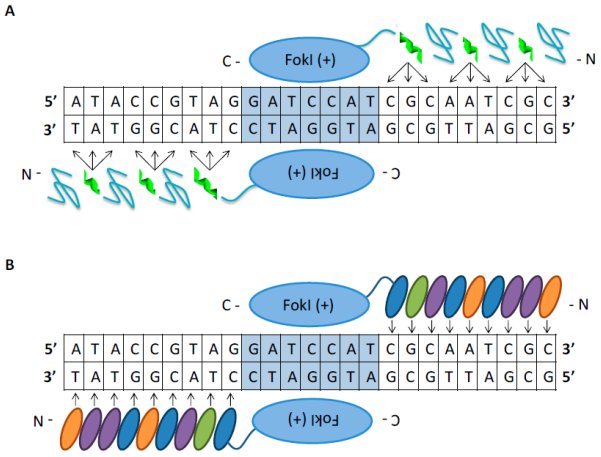


Figure 2: Visual representation of ZFNs and TALENs from *Ates I. et al., Delivery approaches for therapeutic genome editing and challenges.* [16]

1.3.4. CRISP-Cas9

First detected in *E. coli* during the 80's, it was only in the mid 2000's that CRISPR was appropriately described. CRISPR stands for clusters of regularly interspaced short palindromic repeats, which are regular pattern repetitions of a specific DNA sequence, sized between 21 to 37 bp, that appear as clusters in the bacteria chromosome interspaced by unique sequences, the spacer sequences.[17] Nearby these clusters, CRISPR-associated (Cas) genes can be found. Although CRISPR-Cas systems are present in a variety of prokaryotic organisms, it was through the study of bacterial DNA that researchers realized its purpose as an immunological mechanism. It was uncovered that bacteria armed with this type of systems could not only retain fragments of DNA belonging to invading bacteriophages, but also recognize it as foreign DNA templates to identify and disable the bacteriophage in the event of a reinfection (figure 3).[15] This strategy is applied with the help of proteins encoded in Cas genes, the Cas proteins, and produces acquired immunity based on preceding infections that bacteria survived through, in the form of the spacer sequences, referred above, that can be transmitted to the offspring, generating inherited immunity.[13] CRISPR systems are organized into 2 main classes, each divided into three types[18], according to phylogeny, sequence, locus organization and composition.[13] Investigators found that these systems could be manipulated to work in multiple species. The type II system is the famous Cas9 protein

associated system, which was extensively studied and consequently engineered to be used as a genome editing technology.[19]

When bacteria replicate their CRISPR arrays, a complementary RNA molecule containing CRISPR sequences and spacer sequences is produced. This RNA molecule is then cleaved into several fragments each containing a CRISPR RNA (crRNA) and a spacer RNA template. Cas proteins and the trans-activating crRNA (tracrRNA) are encoded on Cas associated genes, near the CRISPR sequences. These tracrRNAs include a segment that links to Cas9 and another that is complementary to CRISPR. Ultimately, the tracrRNA recognizes the crRNA by complementarity enabling the establishment of a ribonucleoprotein complex formed by the dual-RNA, which contains the spacer RNA template and the crRNA linked to the tracrRNA, anchored to a Cas9 protein (figure 4). Each CRISPR/Cas9 complex containing a different template RNA scouts the cytoplasm for foreign DNA complementary to the template sequence. Cas9 has an innate safety checkpoint to assure it is cutting foreign DNA, it cuts at a site preceding a protospacer adjacent motif (PAM) sequence.[19] These sequences are only a few nucleotides long and located downstream of the RNA template sequence, but enough to distinguish foreign DNA, and the presence of an adequate PAM region is imperative for cleavage to occur. Depending on the prokaryotic microorganism that originated a specific Cas protein, the specific nucleotides that compose PAM sequences can vary. The DSB at the specific site where complementarity occurred leads to destruction of invaders' DNA and, therefore, neutralizes the bacteriophages consequences on the bacteria. It is estimated that the cut occurs at three to four nucleotides preceding the PAM sequence. [19]

The possibility of harnessing this system for gene editing in eukaryotic cells is based on manipulating the RNA components anchored to Cas9 to target specific regions. While studying *Streptococus pyogenes* CRISPR/Cas9 system, investigators discovered[20] they could produce a chimeric guideRNA by fusing the crRNA and the tracrRNA, using plasmid engineering. Moreover, instead of the spacer RNA template portion, any previously designed sequence could be added, with an RNA template of 20 nucleotides long. Cas9 will search for a DNA sequence with complementary to the guide RNA target by selecting sequences that match its PAM sequence.[21] When a complementary sequence with an adequate PAM sequence is found by Cas9, both the target sequence and the guide

sequence will be paired upstream the PAM sequence and, if appropriate complementarity is achieved, the nuclease domain of Cas9 will perform a double strand cut.[19,21]

However, experiences conducted in mammalian cells showed that Cas9 specificity in eukaryotic cells was not nearly as high as that observed in bacteria, leading to off-target mutations.[13,22] This was seen as a major setback for the use of this technology in human cells. Several strategies were developed to overcome this problem which will be referred to in the discussion.

The consequences of this discovery were tremendous. Due to this work, chimeric RNAs can now be engineered to match the desired cutting site *a priori* according to the investigator's interest, with a specificity matching the existing TALEN systems, while cheaper and easier to produce. This enabled the creation of mutant models to study disease mechanisms, multiple studies on gene function, fusion proteins, antiviral and antimicrobial applications.[13] These systems made it already to ongoing clinical trials regarding for instance beta-thalassemia (NCT03681951), sickle cell disease (NCT03745287, NCT04774536, NCT04819841), Leber congenital amaurosis (NCT03872479) and hereditary transthyretin amyloidosis (NCT04601051).

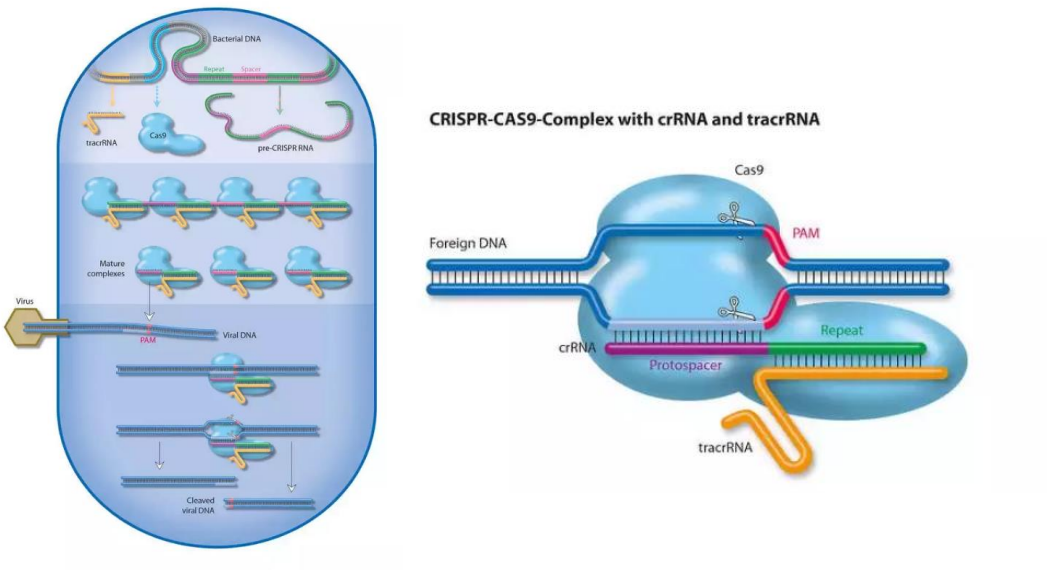
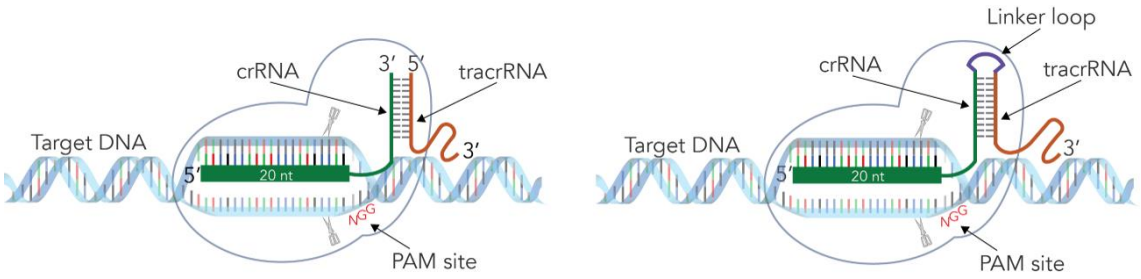


Figure 3 – CRISPR/Cas9 in bacteria; structure of a wild-type and CRISPR/Cas9 system. (Images obtained from *www.mpg.de*)



A. 2-part crRNA:tracrRNA complex B. Single fusion sgRNA trigger

Figure 4 – Structural scheme of constitutive parties in wild-type Cas9 and engineered Cas9 with chimeric RNA. (Image obtained from *www.idtdna.com*)

1.4. Delivery Methods

An effective method to deliver the chosen gene editing system or genetic material to a specific tissue is essential for the success and safety of a gene therapy. Along with the developments of gene editing systems, the search for optimized and safe delivery vectors to carry its components continues to be a main obstacle to tackle.

Viral vectors have been the most used delivery method and its importance in the field has endured through the advancements in editing technologies. In general, this type of approach resorts to an engineered genetic sequence containing a therapeutic gene that is included in the viral vector, which is delivered to the target tissue to be transiently or permanently integrated in the target cell genome. However, efforts to overcome possible immunogenicity and transgene mis-insertion risks led to the development of non-viral approaches.[23]

At the end, the choice of method (viral or not) mainly depends on the type of approach (*in vivo* or *ex vivo*), the target cell/tissue, the editing technology used, the cargo material and the cargo capacity needed.

When discussing viral vectors, three main classes of viruses have been used to develop vectors: retroviruses, adeno-associated viruses and adenoviruses. The following chapter will provide an overview on their contribution to the field. This method is very commonly used to perform gene replacement and gene addition, and the insertion of therapeutic

genes into the cell through viral vectors remains the most studied and widely used approach to gene editing.

Non-viral approaches, although less used, have been emerging throughout the years, and a description of the most pertinent strategies will also be provided, focusing on nanoparticles and electroporation.[23]

1.4.1. Viral Vectors

A viral vector is usually a three-part construct, comprising a viral capsid or envelope, which serves as a container and defines the tissue or cell tropism, a therapeutic DNA sequence, linked to a regulatory cassette that contains a promoter, an enhancer, and auxiliary elements for expression regulation. Each type of viral vector is engineered distinctively and possesses different characteristics. Therefore, the choice of vector depends on the purpose of its use, since some characteristics are desirable for a determined use, but disadvantageous to others.

An analysis of the three most used types of viral vector platforms will follow, focusing on retroviruses, adenoviruses and adeno-associated viruses used for vector production and their applications, focusing on the main differentiating characteristics that influence the choice of platform.

Retroviral Vectors

Retroviruses are single-stranded RNA viruses use their reverse transcriptase to turn the viral RNA into DNA, being advantageous when a permanent genome alteration is desired.[24] The retroviral DNA achieves chromosomic genome integration by gaining access to the nucleus when the nuclear envelope dissolves during cellular division[25], therefore, retroviral vectors mostly target dividing cells. Retroviral vectors can transfer up to 8 kb of exogenous DNA and have been of special importance to gene therapy investigation, since they were the first vectors used in clinical trials. These are also the least immunogenic vectors, regarding innate and adaptative immunity, when compared to adenovirus-based vectors.[26]

Retroviruses can be divided in two main classes, simple retroviruses, such as Murine Leukaemia Virus (MLV), and complex retroviruses, such as Human Immunodeficiency Virus 1 (HIV1), depending on their genomic structure. Most of the knowledge regarding simple retroviruses' genome is based on the study of MLV, and HIV1 is the study model for lentiviruses, a group of complex retroviruses. However, the essential proteins vital for viral viability are transversal to both classes and are encoded into three gene families: *gag* (encoding structural proteins), *env* (encoding envelope proteins) and *pol* (encoding reverse transcriptase enzyme, integrase and proteases). These regions are flanked by Long Terminal Repeats sequences (LTRs), containing the enhancer, promoter, transcription initiation signal, transcription and polyadenylation signal, and control viral genome expression in the host cell. LTRs are separated from protein-coding domains in the viral genome, which allows investigators to replace viral protein-coding genes with a therapeutic gene, while preserving regulatory sequences responsible for maintaining viral integration ability.[27] To build a retroviral vector, the helper genes (*gag, pol* and *env*) are removed and replaced with the therapeutic gene, therefore the virus becomes unable to replicate, since this process depends on the helper proteins encoded in the viral genome, while maintaining its capacity of entering target cells.[28] In order to allow vector production in vitro, helper cell lines have to be used that provide the packaging functions of helper genes. These cell lines contain separate expression constructs driven by non-retroviral regulatory sequences that supply in trans the viral proteins required for viral assembly and packaging.[27]

During the development of retroviral vectors, security concerns were raised when it became clear that replication-competent specimens could emerge through recombination events between the packaging cell genome and the viral construct, and even if rare, its effects could be devastating. Some strategies have been developed to overcome these issues, such as developing new packaging cell lines in which the helper constructs *gag-pro-pol* and *env* are delivered in separate plasmids, using fewer homologous DNA sequences between the vector to be packaged and the helper constructs, LTRs from different viruses, and heterologous promoters. To further increase the safety profile of retroviral vectors, self-inactivating (SIN) vectors have been developed[29] and perfected. SIN vectors contain a deletion of a 3'LTR region that contains

both promoter and enhancer sequences, while a strong promoter from a different virus is added to the 5'LTR, to secure production of high viral titres. When replication occurs, the 3'LTR deletion becomes a 5'LTR deletion, and the added promoter will no longer be on the 5' end, compromising transcriptional activity of the vector in target cells. SIN vectors can also contain an enhanced transcription termination to reduce the possibility of producing oncogenic vectors, which could occur by co-packaging of helper cell's genetic sequences adjacent to the integration site belonging to the packaging cell DNA.[27,30] Retroviruses have naturally evolved to acquire suboptimal transcriptional termination signals, therefore enhanced transcription termination also reduces the probability of read-through into cellular genes following integration of the vector's genetic material on target cells, which could cause up-regulation of protooncogenes.[27,30]

The produced viral vectors can infect target cells in vivo or in vitro, since the viral regulatory cassette and capsid structure are preserved, but they cannot replicate in target cells as the assembled virus lacks viral replication helper sequences. Also, retroviral vectors can undergo pseudotyping, which means that the expression of envelope proteins is controlled to allow choice of tropism and facilitated entry in certain cell types by incorporating foreign viral envelope proteins that are known to concede tropism to a specific tissue type, genetic and chemically modified envelope proteins were also developed to develop direct targeting.[27,31,32]

Retroviral-based vectors, especially gama-retroviral vectors, have already been used in clinical trials, but the development of leukemia in several pediatric patients treated for Wiskott-Aldrich syndrome[33] and SCID[34,35] arose as a major concern for the field. In the case of patients treated for SCID, genetic studies of the malignant cells showed that the gama-retrovirus vector (MLV-based vector) had integrated the genome near promoters of oncogenes, interfering in their expression.[36] It was understood that each virus has preferred integration sites, although not restrictive. Efforts to lead retroviral vectors to targeted integration at "safe harbor" sites have been made, however eliminating the potential for oncogenesis while maintaining effectiveness is a work in progress.[36] A specific group of retroviral vectors have been achieving a better security profile in clinical trials, the lentivirus-based vectors.

Lentiviruses are complex retrovirus that encode non-structural genes related to enhanced replication capability and protein expression regulation[24], however *gag*, *pol*, *env* and LTRs remain the key for maintaining viral function.[28] Lentiviruses can also infect quiescent cells due to their exclusive ability to access the nucleus through an integral nuclear envelope using the nuclear pores, not requiring the cellular division process, which facilitates the lentiviral genome integration process.[37]

Lentiviral vectors are produced according to the safety developments described above for retroviruses in general. Since lentiviruses are complex retroviruses, further alterations must be arranged to increase their safety profile, such as additional removal of accessory virulence factors from the viral genome, supplementary to those found in simple retroviruses.[38] In lentiviral vectors, the ability of using nuclear pores to enter the nucleus of quiescent cells depends on the pre-integration complex (PIC), which is a nucleoprotein constituted of viral genetic material as well as viral and host proteins, classified as an integrase.[39] The lentiviral integrase is derived from the *gag-pol* sequences and is vital to achieve integration of viral DNA, since it recognizes specific LTR sequences and catalyzes the insertion of the LTRs and, consequently, the genetic material between them in the chromosomes. Due to this fact, to reduce the potential risk for insertional mutagenesis, integrase defective mutants were developed.[40,41] Therefore, integrase defective lentiviral vectors allow investigators to decide if they prefer genome integration to be chromosomal or episomal. Also, these vectors can be pseudotyped with the Vesicular Stomatitis Virus glycoprotein G (VSV-G), which recognizes the low-density lipoprotein (LDL) receptor present in every cell, functioning as a pan-tropic envelope protein to broaden vector tropism.[42]

In fact, lentiviral vectors now represent a well-studied and scrutinized delivery platform for gene therapies, with an improved safety profile. The number of clinical trials using this type of vectors is above any other retroviral vectors, and *ex vivo* applications have produced results, i.e., for SCID[43], cerebral adrenoleukodystrophy[44], and Wiskott-Aldrich syndrome[45]. In all clinical trials, no significant toxicity or off-target effects were described, and most patients had a sustained response to treatment. This delivery system is also being used in CAR-T cell therapies, to engineer T-cell receptors to identify

cancer-specific proteins and to fight viral infections, such as COVID-19, but these applications fall out of the context of this work.

Adenoviral Vectors

Adenoviruses are non-enveloped double stranded DNA viruses that can carry up to approximately 36 kb of genetic information and undergo transient episomal genome expression in host cells. These viruses bind to the Coxsackievirus Ad receptor (CAR) and two other co-receptors on the cellular surface, and virions enter the cell by endocytosis. The viruses then escape from endosomes and migrate to the nucleus using the microtubular system. Along this road, viral DNA undergoes uncoating and by the time it reaches the nuclear pore complex, it enters the nucleus in association with several proteins that mask its negative charge and remains stabilized in the nucleoplasm without undergoing chromosomal integration.[37]

The adenoviral genome encodes ~35 proteins and is flanked by hairpin-like inverted terminal repeats (ITRs), which promote primase-independent DNA replication, and a packaging signal.[1,46] Encoded proteins can be grouped in early-phase or late-phase proteins depending on the stage of viral DNA replication they intervene in. Early-phase proteins, such as E1A, E1B, E2, E3 and E4, are transcribed before the initiation of DNA replication. E1A successful transcription is crucial for the transcription of the remainder early-phase proteins, and E1b inactivates p53 to disable cell apoptosis, which is especially relevant for clinical applications. Late-phase proteins, such as L1, L2, L3, L4 and L5, are important for virus assembly and release, and for host cell lysis. The viral capsid is composed of several proteins, some interact with the viral genome and influence genome replication, condensation, and assembly, others are critical for endosomal escape.[47,48]

Adenoviral infection pathway has been extensively studied, mostly using human adenovirus serotypes 5 and 2 (HAd5 and Had2), which are the precursors of modern adenoviral vectors. These underwent several modifications over the years, originating various generations of vectors that show high transduction efficiency and a potential high-titre productivity, representing most viral vectors used in clinical trials. Replacement of E1A, E1B and E3 genes with the therapeutic genetic sequence produce

replication defective vectors, and HEK293 packaging cells lines were developed that provide these gene functions.[1,49,50] However, spontaneous homologous recombination phenomena could produce replication competent virus, and additional deletions of genes E2 and E4 were made, which also decreased cytotoxic T-cell responses, but resulted in insufficient titre production.[1,51] Immunogenicity and cellular toxicity persisted since late-phase proteins were still being produced. Further genome deletions, preserving only ITRs and packaging signal, established "gutless" high cargo capacity vectors, in which native promoters and enhancers of therapeutic genes can be used.[1,52] Replication and packaging functions could be provided by transfection with a helper adenovirus, which contain a marked genome packaging signal recognized and excised by HEK293 enzymes, ensuring that the helper virus genome would not be packaged.[1,53] High-capacity vectors revealed a decrease in immunomodulated unwanted responses, prolonged transduction in host cells and optimized vector production.[1]

Further investigations lead to the development of conditionally replicating adenoviral vectors, by introducing tumor-specific promoters so that the virus will only be able to replicate in cancer cells, causing apoptosis. For example, a partial deletion of E1B, which inactivates p53, disabled the apoptosis deactivating mechanism and, as a result, viral replication was sabotaged in healthy cells while remaining effective in malignant cells that lack a functional p53, leading to apoptosis of cancerous cells only.[1,54] Furthermore, a specific domain of E1A protein, responsible for triggering the release of E2F, was also deleted. E2F acts as a transcription factor that is needed for viral replication in healthy cells but not in malignant cells, since those already produce E2F excessively.[55] Such structural alterations increased selectivity towards malignant cells allowing adenoviral vectors to be used in oncolytic therapies.[1]

Exacerbated host immunogenic responses are the main adverse effect related to adenoviral vectors. Adenoviruses are known to infect humans since the age of 1-3 years old, usually asymptomatically or causing mild upper respiratory tract disease, but rarely affecting the brain and the bladder, and most patients have therefore neutralizing antibodies (NAbs) and antigen-specific T cells against prevalent serotypes, leading to immuno-mediated complications. Ways of overcoming this challenge have been an

object of study, such as the use of less prevalent serotypes non-human infecting adenoviruses.[56]

Nevertheless, the existing immunity to adenovirus can be harnessed in favor of clinical applications that benefit from immunogenic responses, such as vaccines to fight infectious diseases, or cancer, through delivery of immuno-regulatory genes aimed at triggering an immune response towards malignant cells. These strategies will not be approached in this review, however, they are used in clinical trials for targeting Ebola, Influenza, HIV and SARS-Cov2, as well as in clinical trials for prevention of prostate cancer, HPV-associated cancers, colorectal cancer, and pancreatic cancer. Cancer treatments are also being developed using adenoviral vectors to deliver suicide genes, by inducing increased p53 expression or coding pro-drug-converting enzymes.[1,57–59] Adenoviral vectors are also promising vehicles for carrying ZFNs, TALENSs and CRISPR/Cas systems, due to their high cargo capacity. However, the lack of control over immunogenicity must be overcome to rigorously control adenoviral vector engineering and regulate interactions between vectors and the immune system as intended, according to the therapeutic purpose.

Adeno-associated Viral Vectors

Adeno-associated viruses (AAV) were discovered as contaminating agents in adenoviral culture samples and, subsequently observed in human tissues, although not causing any known disease. AAV are single-stranded DNA dependoparvoviruses that depend on the presence of a helper virus, such as an adenovirus, to complete their replication. The replication and expression functions are ensured by adenoviral E1, E2a, E4 proteins and viral associated RNA sequences (non-coding, translation regulator RNA).[1] AAV DNA is constituted of 4 open reading frames (ORFs) flanked by ITRs. The first and second ORFs encode the rep and cap genes, respectively, and the third and fourth ORFs originate assembly-activating protein (AAP), which partakes in capsid assembly, and membrane-associated accessory protein (MAAP), which function is still under investiagtion.[1,60] The ITRs function as replication primers and packaging signal and offer recombination ability intra and inter-molecule to stabilize episomal genome forming circular or concatemerized dsDNA.[1,61] The virus enters the cell by endocytosis, and capsid proteins,

which contain nuclear localization signals, guide the viral DNA to its destination, through a mechanism that is still being studied.[1,62]

Although they have a limited cargo capacity of ~4 kb, AAV vectors present interesting characteristics for their use in gene therapies, such as ability to infect dividing and quiescent cells, preferential episomal genome maintenance, and use of tissue specific promoters to achieve precise tissue or cell tropism. Different serotypes have distinct tissue and cell tropism, and one of them, AAV9, is known to bypass the blood-brain barrier, which is potentially useful for clinical applications. AAV vectors are considered the least immunogenic of all available viral vectors and increasing their low cargo capacity remains the main challenge to optimize a cost-effective option. To overcome their restricted capacity, a few strategies are being studied, such as dual vector systems using techniques based on ITRs recombination events between complementary vector genomes, encoding truncated but functional proteins, RNA and protein trans-splicing, and homology strategies.[1,63–69] However, in depth understanding of the contribute of ITRs to transgene expression is yet to be reached.[1]

Although AAV vectors are the least immunogenic, immunomodulated responses still play an essential role in decreasing effectiveness in humans, in part due to previous exposure. The capsid's role in the infection process is not yet fully understood, but it plays an important role in vectors' immunogenicity.[70] The capsid proteins, the viral genome and the transgene-encoded protein remain the main sources of immunogenicity.[71] The dsRNAs which are thought to be a result of the ITRs role as promoters, producing transcripts on the minus strand, are also a source of immunogenicity.[1,71,72] Additional investigations are needed to fully understand how host and vector individually contribute to trigger this type of responses.

A better understanding of structural components' functionalities and interactions with the host are required to fully manipulate the vector in order to decrease immunogenicity and improve effectiveness.

Production of AAV vectors mostly resorts to HEK293 packaging cell lines transfected with a helper adenovirus that provides the E1a and E1b genes, and a plasmid containing the therapeutic gene. Since this approach only meets the required viral titre levels for 2-3

days, eventually losing viability, stable mammalian cell lines were developed to increase productivity.[1] Each immortalized cell line was engineered to produce a specific vector construct and were transfected with an AAV vector containing *rep-cap* and the transgene, which would be intrinsically expressed along with the remaining construct components, so helper plasmids were no longer required.[1,73] With each cell line being produced only for a specific construct, an alternative approach was tried by transfecting the cells with AAV/Ad hybrid vectors, to deliver the transgene and Ad elements to provide strong expression of *rep-cap*.[1,74,75] Besides producing higher viral titres, these approaches limit the chances of contamination by other viruses.[1] However, the concern with wrongful packaging of cellular immortalizing elements as a possible source of oncogenes is real, and several systems have been developed to determine their presence in AAV vector produced by these means.[1]

Regarding capsid manipulation, several engineering techniques can be used to attempt production of least immunogenic capsids and choice of tissue-tropism. Resorting to the study of natural occurring capsids with desirable characteristics, rational design can attach tissue-specific peptides to the engineered capsids.[46] Directed evolution resorting to mutagenesis and combinations between existent capsids with required properties, as well as computational modelling (*in silico* approach) are also applied.[46]

Currently, AAVs are the leading delivery platform for gene therapies, and have produced a total of three FDA approved and already commercialized gene therapies: Glybera (alipogene tiparvovec), Luxturna (voretigene neparvovec-rzyl) and Zolgensma (onasemnogene abeparvovec). These therapies are based on gene replacement and treat recessive monogenic diseases. *Glybera* is a treatment for lipoprotein lipase deficiency and uses an AAV-1-based vector construct to deliver the lipoprotein lipase gene[76]. It was the first AAV-based gene therapy to gain EMA approval. *Luxturna* treats Leber's congenital amaurosis, using an AAV2-based vector to deliver the retinoid isomerohydrolase RPE65 gene.[77] *Zolgensma* is used to deliver a functional SMN1 gene to patients with SMA, requiring only one administration.[78] All these approved therapies have a challenging factor in common, the price, which varies between $425.000 per eye, for *Luxturna*, and $2,125 million for *Zolgensma*. Furthermore, *Glybera* was taken off the

market due to being an extremely expensive treatment for a rare genetic disease (1 per million). [1]

Nowadays, the most promising feature of these vectors, and that will certain be the center of upcoming clinical trials, is their ability to carry CRISPR/Cas systems components.

1.4.2. Non-viral Techniques

Most clinical trials regarding gene therapies resort to viral vectors to carry the therapeutic genetic material into cells, but challenges associated with immunogenicity remain. With the development of new gene editing technologies, transporting all components to the target cell requires enlarged cargo capacity. To overcome the limitations of viral delivery systems, non-viral alternatives continue to be developed. Wild-type viruses are already optimized to achieve successful genome delivery into cells and naturally evolved to infect human organisms, which can be harnessed to engineer viral vectors. Non-viral delivery vectors include materials that are products of molecular engineering, or physical strategies and, although more difficult to optimize, generally present least immunogenic problems since they are completely artificial. In this review, a few non-viral delivery methods were chosen to represent several types of delivery. Regarding physical methods, electroporation, hydrodynamic delivery and microinjection will be discussed. Non-physical delivery methods will be represented by lipid nanoparticle mediated delivery, and cell penetrating peptides. Although these methods are reviewed individually, their conjoined evolution has collided at some points, and hybrid systems are also being studied as a way of harnessing different desirable characteristics of each method in combined systems.

Electroporation

Gene therapy delivery by electroporation is based on a physical transformation of the host cellular membrane caused by exposure to electric fields of proper strength, duration, form and number, causing the transient development of pore-like gaps in the membrane through which small or large molecules can enter the cell.[79] Obtaining precise control over these variables is very complex, especially *in vivo*, due to

unpredictable variations of the organism and the possibility of multisystemic consequences, which limits electroporation based delivery to *ex vivo* applications.

Achieving a successful electroporation process results in high cell viability, which greatly depends on precise permeabilization of cellular membranes. Unwarranted permeabilization can lead to membrane continuity dissolution, and therefore cellular death. On the contrary, deficient permeabilization will lead to low transfection efficiency.[79] Electroporation-based delivery techniques use buffers to help maintain membrane integrity. These are artificially fabricated solutions that can be saline-based, phosphate-based, HEPES-based, or cell-culture-media based, with variable conductivity, which depends on the added salts and the final osmolality (modified by osmotic agents, such as sugar or an inert protein).[79]

A few hurdles are still hard to tackle and limit a wider use of electroporation, such as the limited number of cells that are effectively electroporated, resulting in low efficiency of cytoplasm delivery and incorporation of exogenous components, which leads to a low rate of gene-corrected cells.[16] Another issue is cell viability after the procedure, which appears to be low in many cases.[80] The main goal is to improve transfection rates while increasing cell viability, either by adjusting the electroporation vessel design, improving the buffers, or using nucleofection, for example.[16] Regarding vessel improvement, using tube electroporation leads to significantly higher rates of transduced cells, unlike using cuvettes (the mostly used vessel).[81] This might be because of the uneven distribution of electric charge possibly due to the concave meniscus formed on the cuvette, concentrating a part of the solution around it, and the formation of air bubbles, creating charge differentials, resulting either in unwarranted or deficient permeabilization.[81] By using a tube, there is an even distribution of the solution, and by placing the electrodes on both ends and compressing the solution within the tube and the electrodes, the surface solution distribution is even.[81] Also, a smaller area of the electrodes is in contact with the solution, minimizing air bubbles.[81] This method produced high rates of transfection with a CRISPR/Cas9 based system in several cell types (74-90.7%), resulting in high cell viability, higher rates of HDR occurrence, and high editing efficiency in different cell types.[81] Concerning buffers improvement, it has been shown that the solution components can affect the effectiveness of electroporation, i.e., in an optimal

concentration, Mg^{2+} can act as an enzymatic co-factor increasing cell viability, and should be included in buffer solutions.[79]

Nucleofection is an electroporation-based method to deliver DNA directly into the cell nucleus, also known as Nucleofector (Trade Mark of Lonza Bioscience), which can transfect non-dividing cells without depending on cellular division to gain access to the nucleus[82], and is being studied as a possible option to retroviral vectors. [83,84]

This technique is mostly employed *ex vivo* in the modification of autologous CD34+ cells, to deliver the gene editing system[16], and has made it into a few clinical trials regarding HIV86, sickle cell anemia and beta-thalassemia (NCT03655678, NCT03745287)[16] and anticancer therapies[86].

Electroporation remains under investigation as a promising delivery method to *ex vivo* gene editing.

Hydrodynamic Delivery

Hydrodynamic delivery is a physical delivery method based on the administration of a high volume of a solution containing a high concentration of genetic material directly into the circulatory system. Since the blood is non-compressible, the increase in vascular pressure leads to a transitory higher permeability of cellular membranes, therefore enabling transfer of the exogenous DNA into the surrounding cells and tissues.[16,87,87]

This technique is especially interesting for targeting liver cells. Due to the non-compressible character of liquids, injection of a DNA solution in the inferior vena cava directs the high-volume of liquids straight through the heart and backward flow happens, overloading the hepatic circulation and forcing the injected DNA into hepatocytes.[16] This rational can potentially be applied to any body part through a selection of the most appropriate circulation pathway to target a specific organ. However, the level of congestion caused to the cardiovascular system entails serious acute side-effects. This could be improved using catheters and circulation occlusion in target organs, for example.[16]

This method was developed and is being used in rodents for in vivo genome editing, generally to deliver naked plasmids, and has produced interesting results in hereditary

tyrosinemia, hemophilia A, and rodents positive for Hepatitis B Virus, as well as in the study of various disease mechanisms.[16] However, safety concerns have blocked its applications in clinical trials.

Microinjection

The microinjection technique consists of inserting the desired gene therapy material into a targeted cell through a microneedle, using a microscope.[88] The advantages of this approach is that targeting different locations within the cell is possible (cytoplasm versus nucleus) with extremely high transfection efficiency, due to the bypass of extracellular and membrane obstacles, no cargo capacity limit and control over the amount of product administered.[88] Usually, microinjection is used to deliver nucleic acids, which can be done by delivering DNA or mRNA directly to the nucleus, or delivering mRNA to the cytoplasm.[88]

This method is the textbook approach to deliver CRISPR/Cas systems, and the most commonly used approach is to deliver the mRNA encoding Cas9 and the guide RNA to the cytoplasm. [88] However, cell viability may be compromised by the microinjection process. The application of this procedure to animal models' zygotes is widely used to produce animal models of disease, although its application in human cells is yet to be investigated. This technique has been around for a while and will not be further discussed here. However, a recent study compared electroporation with microinjection to introduce a CRISPR/Cas9 system into porcine embryos and concluded that electroporation was more reliable when the major interest is to maintain embryo's viability and transgene expression within desired levels[89], as in human embryos.

Lipid Nanoparticles

Lipids are naturally occurring and biodegradable, and gene therapies' delivery usually resorts to cationic lipids or ionizable lipids. Methods using cationic lipids rely on the positive charge of these nanoparticles, which facilitates binding to negatively charged molecules, such as nucleic acids, forming cationic liposomes. These allow delivery of the encapsulated nucleic acids by fusion with cellular membranes, while shielding the cargo from degradation.[90] However, toxicity and low transfection effectiveness led to the development of ionizable lipids, which are neutral at physiological pH, facilitating

delivery, and can be positively charged to facilitate liposome formation.[23] Cholesterol, phospholipids and polyethylene glycol can be inserted in nanoparticle vectors to improve stability, delivery efficiency, tolerability and biodistribution.[91] Other lipid-based vectors were developed, such as gemini surfactants and lipidoids. Gemini surfactants appear to have a lower toxicity due to the reduced amount of vector needed to perform delivery related to their high solubility.[23] Lipidoids are artificially synthetized lipid-like particles and have showed promising results.[23] Lipid-based nanoparticles can deliver the cargo material without causing genomic integration.

Lipid nanoparticles are being used to deliver CRISPR/Cas systems. However, if a repair template needs to be provided for HDR, it has to be delivered in a separate nanoparticle, other than the nanoparticle carrying Cas 9.[12] The delivery cargo can be either genetic material (plasmid with guide RNA and Cas mRNA) or ribonucleoprotein complexes (Cas9 protein and guide RNA complexes, which are highly anionic and approached as nucleic acids in delivery).[88] This approach has been used to deliver CRISPR/Cas9 systems and ZFNs to mice, *in vivo*, with promising results[91–93], and a recent study concluded that a further developed system is significantly more efficient than the FDA-approved MC-3 lipid nanoparticle protein used in Onpattro[23], an FDA approved siRNA drug, allowing liver targeted gene editing.[94]

Production of COVID mRNA vaccines exploited lipid nanoparticles to deliver of antigen mRNA[91,95,96], and is also being used in the development of other vaccines, cancer immunotherapies, enzyme replacement therapies delivery and, in the field of gene therapy.[91] Applications in human gene therapy are the next step for this method of delivery.

Cell-penetrating Peptides

Cell-penetrating peptides are small sequences of amino acids with innate polycationic, amphipathic or non-polar nature, and their use as delivery vectors relies on their association with the cargo material to facilitate cell entry.[88] Different classes and combinations of peptides can be used to achieve some level of cellular tropism, and these particles are biodegradable and biocompatible.[23,88] Cell-penetrating peptides have been exploited to delivery gene therapies. These are uncharged particles

constituted of peptides and nucleic acids linked through covalent bonds that show a better capability of resisting degradation.[23] However, as for lipid nanoparticles, it is hard to decrease endosomal entrapment. Dendrimer designs, constructed using amino acids, contain positively charged groups that improve interaction with genetic cargos and seem to enhance endosome escape capability. Other particles, such as lipids, can also be included in dendrimer designs to facilitate cell entry.[23] Tissue specific and wide tropism peptides have been developed and bring a new development road for clinical applications.[97]

In the field of gene therapy, these vectors have been used together with siRNA technologies.[97] An interesting approach conjugated an adenoviral vector with cell penetrating peptides to bypass the need for interaction with CAR receptors, essential for adenoviruses cellular entry, and showed enhanced transduction on CAR devoid cells.[97,98] Cas9 and guide RNA delivery showed an increased transfection efficiency, with decreased cellular toxicity and off-target mutations.[97,99–101] Other applications to improve viral vectors were also developed, and plasmid delivery has also benefited from using cell-penetrating peptides, and a multiplicity of investigations were developed to understand the full potential of these approach.[97]

2. METHODS

2.1. Study Characterization and Relevance

The present study is a systematic review on recent advances in the field of gene therapy, as it uses a previously defined approach (PRISMA approach) to integrate pertinent studies, conducted under explicit and reproducible methodology.

Gene therapy has been under thorough scrutiny since it promises to change the natural course of several uncurable diseases. The concerns with safety and ethical issues in previous clinical trials have caused a few setbacks. In recent years, advances in this field of expertise allowed an increase in the number of clinical trials with gene therapy technology and led to approval of effective gene therapies wherein the benefits outweigh the risks.

In this work, the main concern is to understand which of the available gene-editing technologies are used in clinical trials, and to recognize new advances in scientific knowledge.

In current medical practice, the analysis of recently completed clinical trials, as well as FDA-approved treatments, is a necessary tool to update doctors on this subject and improve their ability to guide patients about emerging treatment options.

2.2. Search Strategy, Selection Process and Data Extraction

A search was conducted on clinicaltrials.gov in March, 2021. The following limits were applied when using the website's search engine: "with results" + "completed". Limits granted a preliminary selection of clinical trials that were already finalized to increase the possibilities of finding published articles with robust conclusions instead of premature results and analysis. Individual searches were conducted using the following

terms: “gene therapy”, “viral vectors”, “adeno-associated virus”, “adenovirus”, “CRISP + Cas”, “TALEN”, “ZFN”, “RNAi”, “retrovirus + gene therapy” and “lentivirus + gene therapy”.

The full study records from the “Study Record Detail” section of each trial file were downloaded in a .zip file through the platform’s downloading tool - “Download content for analysis” - in .xml format. Subsequently, a script developed using UiPath Studio was used to screen the .xml files for the same keywords used in the previous search. The software was programmed to collect the data into an Excel Office 365 for Windows file and filter it by keyword found in the .xml file screening, associating the respective title and NCT and distinguish trials by date of initial and last update posted.

Additionally, two reviews were consulted to obtain a list of available gene therapies worldwide.[102,103]

When consulting a few of the reviews referenced in this work, it was clear that the obtained list of clinical trials, although extensive, failed to contain several recent investigations.

A new search was conducted on clinicaltrials.gov in August 2021, with the same keywords but devoid of the filters, producing a new list of clinical trials.

According to the framework, a high-volume sample was gathered. Both lists resulting from the search, were merged, and repeated clinical trials were eliminated.

A total of 326 clinical trials were admitted for screening considering the title or the information found on the study page on clinicaltrials.gov, and when necessary, additional research.

The information was synthesized by using tables and graphics created with Excel Office 365 for Windows. The following information was extracted from the articles analyzed: corresponding clinical trial by NCT, title, target disease, gene therapy techniques applied (vector and editing method).

3. RESULTS

The initial pool included 326 studies from which 249 met the inclusion criteria. Included trials were analyzed individually based on the title or information present on the study page at *clinicaltrials.gov* and 83 studies were excluded. A total of 206 studies were admitted to this review.

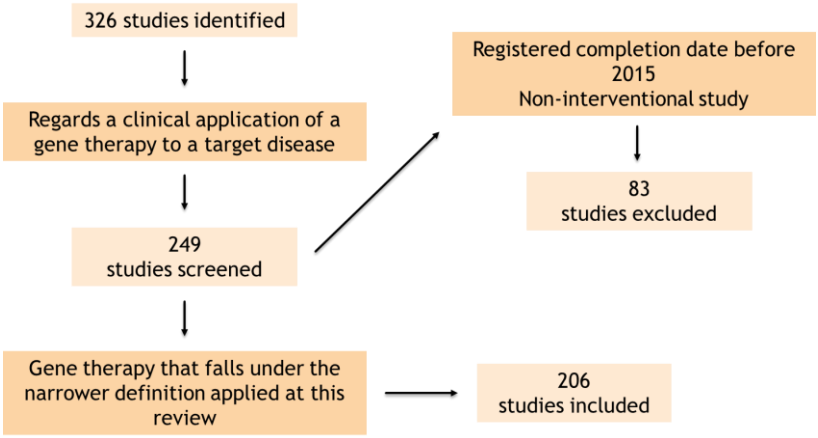


Figure 5 – Flowchart (based on PRISMA chart) summarizing the search phases.

The goal of this search was to obtain a representative sample of the clinical applications in gene therapy landscape. During data collection, the final sample was categorized and listed on table 1.

NCT	TITLE	TARGET DISEASE
NCT00076557	Safety of a New Type of Treatment Called Gene Transfer for the Treatment of Severe Hemophilia B	Hemophilia B
NCT00151216	Safety Study of a Gene Transfer Vector for Children With Late Infantile Neuronal Ceroid Lipofuscinosis	Batten Disease
NCT00377416	Experimental Gene Transfer Procedure to Treat Alpha 1-Antitrypsin Deficiency	Alpha 1-Antitrypsin Deficiency
NCT00408590	Recombinant Measles Virus Vaccine Therapy and Oncolytic Virus Therapy in Treating Patients With Progressive, Recurrent, or Refractory Ovarian Epithelial Cancer or Primary Peritoneal Cancer	Ovarian Cancer|Primary Peritoneal Cavity Cancer
NCT00430768	Experimental Gene Transfer Procedure to Treat Alpha 1-Antitrypsin (AAT) Deficiency	Alpha 1-Antitrypsin Deficiency
NCT00450814	Vaccine Therapy With or Without Cyclophosphamide in Treating Patients With Recurrent or Refractory Multiple Myeloma	Recurrent Plasma Cell Myeloma
NCT00481546	Phase I Trial of Gene Vector to Patients With Retinal Disease Due to RPE65 Mutations	Leber Congenital Amaurosis
NCT00516477	Safety Study in Subjects With Leber Congenital Amaurosis	Leber Congenital Amaurosis
NCT00534703	Investigation of the Safety and Feasibility of AAV1/SERCA2a Gene Transfer in Patients With Chronic Heart Failure	Chronic Heart Failure
NCT00598481	ADA Gene Transfer Into Hematopoietic Stem/Progenitor Cells for the Treatment of ADA-SCID	Adenosine Deaminase Deficiency Severe Combined Immunodeficiencies
NCT00643890	Study of AAV-GAD Gene Transfer Into the Subthalamic Nucleus for Parkinson's Disease	Parkinson's Disease
NCT00749957	Phase 1/2 Safety and Efficacy Study of AAV-RPE65 Vector to Treat Leber Congenital Amaurosis	Leber Congenital Amaurosis
NCT00794508	MND-ADA Transduction of CD34+ Cells From Children With ADA-SCID	Adenosine Deaminase Deficiency Severe Combined Immunodeficiencies
NCT00821340	Clinical Trial of Gene Therapy for Leber Congenital Amaurosis Caused by RPE65 Mutations	Leber Congenital Amaurosis
NCT00876863	Randomized, Controlled Study Evaluating CERE-110 in Subjects With Mild to Moderate Alzheimer's Disease	Alzheimer's Disease
NCT00976352	Safety Study of Recombinant Adeno-Associated Virus Acid Alpha-Glucosidase to Treat Pompe Disease	Pompe Disease
NCT00979238	Dose-Escalation Study Of A Self Complementary Adeno-Associated Viral Vector For Gene Transfer in Hemophilia B	Hemophilia B
NCT00985517	Safety and Efficacy of CERE-120 in Subjects With Parkinson's Disease	Idiopathic Parkinson's Disease
NCT00999609	Safety and Efficacy Study in Subjects With Leber Congenital Amaurosis	Leber Congenital Amaurosis
NCT01024998	Safety and Tolerability Study of AAV2-sFLT01 in Patients With Neovascular Age-Related Macular Degeneration (AMD)	Age-Related Maculopathies|Retinal Degeneration|Retinal Neovascularization

NCT01054339	Safety & Efficacy Study of rAAV1-CB-hAAT for Alpha-1 Antitrypsin Deficiency	Alpha-1 Antitrypsin Deficiency
NCT01161576	Safety Study of a Gene Transfer Vector (Rh.10) for Children With Late Infantile Neuronal Ceroid Lipofuscinosis (LINCL)	Batten Disease
NCT01267422	Safety and Efficacy Study of rAAV2-ND4 Treatment of Leber Hereditary Optic Neuropathy (LHON)	Leber Optic Neuropathy
NCT01306019	Lentiviral Gene Transfer for Treatment of Children Older Than Two Years of Age With X-Linked Severe Combined Immunodeficiency (XSCID)	X-Linked Severe Combined Immune Deficiency
NCT01380990	Lentiviral (LV) Gene Therapy for Adenosine Deaminase (ADA) Deficiency	Adenosine Deaminase Deficiency Severe Combined Immunodeficiencies
NCT01395641	A Phase I/II Clinical Trial for Treatment of Aromatic L-amino Acid Decarboxylase (AADC) Deficiency Using AAV2-hAADC	Aromatic L-amino Acid Decarboxylase Deficiency
NCT01414985	AAVRh.10 Administered to Children With Late Infantile Neuronal Ceroid Lipofuscinosis	Batten Disease
NCT01438112	Efficacy Study of Recombinant Adenovirus for Non Muscle Invasive Bladder Cancer	Bladder Cancer
NCT01461213	Gene Therapy for Blindness Caused by Choroideremia	Choroideremia
NCT01482195	Trial of Subretinal Injection of (rAAV2-VMD2-hMERTK)	Retinitis Pigmentosa
NCT01494805	Safety and Efficacy Study of rAAV.sFlt-1 in Patients With Exudative Age-Related Macular Degeneration	Age-related Maculopathies|Retinal Degeneration|Retinal Neovascularization
NCT01519349	Follistatin Gene Transfer to Patients With Becker Muscular Dystrophy and Sporadic Inclusion Body Myositis	Becker Muscular Dystrophy|Sporadic Inclusion Body Myositis
NCT01620801	Hemophilia B Gene Therapy With AAV8 Vector	Hemophilia B
NCT01621581	AAV2-GDNF for Advanced Parkinson s Disease	Parkinson's Disease
NCT01643330	A Study of Genetically Targeted Enzyme Replacement Therapy for Advanced Heart Failure	Heart Failure
NCT01687608	Open-Label Single Ascending Dose of Adeno-associated Virus Serotype 8 Factor IX Gene Therapy in Adults With Hemophilia B	Hemophilia B
NCT01734850	Safety Study of a Dual Anti-HIV Gene Transfer Construct to Treat HIV-1 Infection	Human Immunodeficiency Virus 1
NCT01787994	Redirected MazF-CD4 Autologous T Cells for HIV Gene Therapy	Human Immunodeficiency Virus 1
NCT01801709	Intracerebral Gene Therapy for Children With Early Onset Forms of Metachromatic Leukodystrophy	Metachromatic Leukodystrophy

NCT01811992	Combined Cytotoxic and Immune-Stimulatory Therapy for Glioma	Malignant Glioma|Glioblastoma Multiforme
NCT01913106	HSV-tk + Valacyclovir Therapy in Combination With Brachytherapy for Recurrent Prostate Cancer	Prostatic Cancer
NCT01961063	Gene Therapy After Frontline Chemotherapy in Treating Patients With AIDS-Related Non-Hodgkin Lymphoma	AIDS-related Non-Hodgkin Lymphoma
NCT01966887	AAV1-CMV-Serca2a GENe Therapy Trial in Heart Failure	Heart Failure
NCT01973543	Safety Study of AADC Gene Therapy (VY-AADC01) for Parkinson's Disease	Parkinson's Disease
NCT02077361	An Open Label Clinical Trial of Retinal Gene Therapy for Choroideremia	Choroideremia
NCT02122952	Gene Transfer Clinical Trial for Spinal Muscular Atrophy Type 1	Spinal Muscular Atrophy
NCT02140554	A Study Evaluating the Safety and Efficacy of bb1111 in Severe Sickle Cell Disease	Sickle Cell Disease
NCT02143804	Safety and Efficacy of CG0070 Oncolytic Virus Regimen in Patients With High Grade Non-Muscle Invasive Bladder Cancer	Bladder Cancer
NCT02161380	Safety Study of an Adeno-associated Virus Vector for Gene Therapy of Leber's Hereditary Optic Neuropathy	Leber Optic Neuropathy
NCT02234934	Study of Gene Therapy Using a Lentiviral Vector to Treat X-linked Chronic Granulomatous Disease	Granulomatous Disease
NCT02240407	Re-administration of Intramuscular AAV9 in Patients With Late-Onset Pompe Disease	Pompe Disease
NCT02317887	Study of RS1 Ocular Gene Transfer for X-linked Retinoschisis	X-Linked Retinoschisis
NCT02337985	Gene Therapy and Combination Chemotherapy in Treating Patients With AIDS-Related Non-Hodgkin Lymphoma	AIDS Related Non-Hodgkin Lymphoma
NCT02341807	Safety and Dose Escalation Study of AAV2-hCHM in Subjects With CHM (Choroideremia) Gene Mutations	Choroideremia
NCT02346422	A Phase 1/2 Study of High-dose Genetically Targeted Enzyme Replacement Therapy for Advanced Heart Failure	Heart Failure
NCT02354781	Clinical Intramuscular Gene Transfer of rAAV1.CMV.huFollistatin344 Trial to Patients With Duchenne Muscular Dystrophy	Duchenne Muscular Dystrophy
NCT02376816	Clinical Intramuscular Gene Transfer Trial of rAAVrh74.MCK.Micro-Dystrophin to Patients With Duchenne Muscular Dystrophy	Duchenne Muscular Dystrophy
NCT02378922	Gene-Modified HIV-Protected Stem Cell Transplant in Treating Patients With HIV-Associated Lymphoma	AIDS-related Lymphoma
NCT02396342	Trial of AAV5-hFIX in Severe or Moderately Severe Hemophilia B	Hemophilia B
NCT02407678	REP1 Gene Replacement Therapy for Choroideremia	Choroideremia
NCT02416622	Safety and Efficacy of rAAV-hRS1 in Patients With X-linked Retinoschisis (XLRS)	X-linked Retinoschisis
NCT02418598	AADC Gene Therapy for Parkinson's Disease	Parkinson’s Disease

NCT02446249	Safety of a Single Administration of AAV2hAQP1, an Adeno-Associated Viral Vector Encoding Human Aquaporin-1 to One Parotid Salivary Gland in People With Irradiation-Induced Parotid Salivary Hypofunction	Radiation Induced Xerostomia|Salivary Hypofunction
NCT02484092	A Gene Therapy Study for Hemophilia B	Hemophilia B
NCT02553135	Choroideremia Gene Therapy Clinical Trial	Choroideremia
NCT02576795	Gene Therapy Study in Severe Haemophilia A Patients (270-201)	Haemophilia A
NCT02599922	Safety and Efficacy Trial of AAV Gene Therapy in Patients With CNGB3 Achromatopsia	Achromatopsia
NCT02618915	Safety and Dose Finding Study of DTX101 (AAVrh10FIX) in Adults With Moderate/Severe to Severe Hemophilia B	Hemophilia B
NCT02651675	A Gene Therapy Study for Homozygous Familial Hypercholesterolemia (HoFH)	Homozygous Familial Hypercholesterolemia
NCT02652767	Efficacy Study of GS010 for the Treatment of Vision Loss up to 6 Months From Onset in LHON Due to the ND4 Mutation	Leber Congenital Amaurosis
NCT02652780	Efficacy Study of GS010 for Treatment of Vision Loss From 7 Months to 1 Year From Onset in LHON Due to the ND4 Mutation (REVERSE)	Leber Congenital Amaurosis
NCT02671539	THOR - Choroideremia Gene Therapy Trial	Choroideremia
NCT02695160	Ascending Dose Study of Genome Editing by Zinc Finger Nuclease Therapeutic SB-FIX in Subjects With Severe Hemophilia B	Hemophilia B
NCT02702115	Ascending Dose Study of Genome Editing by the Zinc Finger Nuclease (ZFN) Therapeutic SB-318 in Subjects With MPS I	Mucopolysaccharidosis
NCT02704325	Gene Transfer Clinical Trial for Duchenne Muscular Dystrophy Using rAAVrh74.MCK.GALGT2	Duchenne Muscular Dystrophy
NCT02710500	rAAVrh74.MHCK7.DYSF.DV for Treatment of Dysferlinopathies	Dysferlinopathy
NCT02716246	Phase I/II Gene Transfer Clinical Trial of scAAV9.U1a.hSGSH	Mucopolysaccharidosis
NCT02725580	Gene Therapy for Children With Variant Late Infantile Neuronal Ceroid Lipofuscinosis 6 (vLINCL6) Disease	Variant Late-Infantile Neuronal Ceroid Lipofuscinosis
NCT02727764	A Single Dose Clinical Trial to Study the Safety of ART-I02 in Patients With Arthritis	Osteoarthritis
NCT02773849	INSTILADRINÂ® in Patients With Bacillus Calmette-Guerin (BCG) Unresponsive Non-Muscle Invasive Bladder Cancer (NMIBC)	Bladder Cancer
NCT02781480	Clinical Trial of Gene Therapy for the Treatment of Leber Congenital Amaurosis (LCA)	Leber Congenital Amaurosis
NCT02790723	Safety of Intra-Articular Sc-rAAV2.5IL-1Ra in Subjects With Moderate Knee OA	Osteoarthritis
NCT02797470	Gene Therapy in Treating Patients With Human Immunodeficiency Virus-Related Lymphoma Receiving Stem Cell Transplant	HIV-related Lymphoma

NCT02831933	Trial of Radiation and Gene Therapy Before Nivolumab for Metastatic Non-Small Cell Lung Carcinoma and Uveal Melanoma	Lung Cancer
NCT02852213	A Single-Stage, Adaptive, Open-label, Dose Escalation Safety and Efficacy Study of AADC Deficiency in Pediatric Patients	Aromatic Amino Acid Decarboxylase Deficiency
NCT02926066	A Clinical Trial for Treatment of Aromatic L-amino Acid Decarboxylase (AADC) Deficiency Using AAV2-hAADC - An Expansion	Aromatic Amino Acid Decarboxylase Deficiency
NCT02935517	Safety and Efficacy Trial of AAV Gene Therapy in Patients With CNGA3 Achromatopsia	Achromatopsia
NCT02991144	Safety and Dose-Finding Study of DTX301 (scAAV8OTC) in Adults With Late-Onset OTC Deficiency	Ornithine Transcarbamylase Deficiency
NCT03001310	Gene Therapy for Achromatopsia (CNGB3)	Achromatopsia
NCT03001830	Gene Therapy for Haemophilia A.	Hemophilia A
NCT03003533	A Gene Transfer Study for Hemophilia A	Hemophilia A
NCT03041324	Ascending Dose Study of Genome Editing by the Zinc Finger Nuclease (ZFN) Therapeutic SB-913 in Subjects With MPS II	Mucopolysaccharidosis
NCT03061201	A Study of Recombinant AAV2/6 Human Factor 8 Gene Therapy SB-525 (PF-07055480) in Subjects With Severe Hemophilia A	Hemophilia A
NCT03065192	Safety and Efficacy Study of VY-AADC01 for Advanced Parkinson's Disease	Parkinson's Disease
NCT03066258	Safety and Tolerability of RGX-314 Gene Therapy for Neovascular AMD Trial	Neovascular Age-related Macular Degeneration
NCT03116113	A Clinical Trial of Retinal Gene Therapy for X-linked Retinitis Pigmentosa Using BIIB112	X-Linked Retinitis Pigmentosa
NCT03153293	A Single Intravitreal Injection of rAAV2-ND4 for the Treatment of Leber's Hereditary Optic Neuropathy	Leber Optic Neuropathy
NCT03173521	Gene Therapy in Patients With Mucopolysaccharidosis Disease	Mucopolysaccharidosis
NCT03199469	Gene Transfer Clinical Study in X-Linked Myotubular Myopathy	X-Linked Myotubular Myopathy
NCT03223194	Gene Transfer Clinical Study in Crigler-Najjar Syndrome	Crigler-Najjar Syndrome
NCT03252847	Gene Therapy for X-linked Retinitis Pigmentosa (XLRP) Retinitis Pigmentosa GTPase Regulator (RPGR)	X-Linked Retinitis Pigmentosa
NCT03282656	Gene Transfer for Sickle Cell Disease	Sickle Cell Disease
NCT03293524	Efficacy & Safety Study of Bilateral IVT Injection of GS010 in LHON Subjects Due to the ND4 Mutation for up to 1 Year	Leber Optic Neuropathy
NCT03300453	Intracerebral Gene Therapy in Children With Sanfilippo Type B Syndrome	Mucopolysaccharidosis
NCT03306277	Gene Replacement Therapy Clinical Trial for Participants With Spinal Muscular Atrophy Type 1	Spinal Muscular Atrophy
NCT03307980	Long-term Safety and Efficacy Study and Dose-Escalation Substudy of PF 06838435 in Individuals With Hemophilia B	Hemophilia B

NCT03315182	Gene Transfer Clinical Trial for Mucopolysaccharidosis (MPS) IIIB	Mucopolysaccharidosis
NCT03316560	Safety and Efficacy of rAAV2tYF-GRK1-RPGR in Subjects With X-linked Retinitis Pigmentosa Caused by RPGR Mutations	X-Linked Retinitis Pigmentosa
NCT03326336	Dose-escalation Study to Evaluate the Safety and Tolerability of GS030 in Subjects With Retinitis Pigmentosa	Non-syndromic Retinitis Pigmentosa
NCT03328130	Safety and Efficacy Study in Patients With Retinitis Pigmentosa Due to Mutations in PDE6B Gene	Retinitis Pigmentosa
NCT03333590	Gene Transfer Clinical Trial to Deliver rAAVrh74.MCK.GALGT2 for Duchenne Muscular Dystrophy	Duchenne Muscular Dystrophy
NCT03362502	A Study to Evaluate the Safety and Tolerability of PF-06939926 Gene Therapy in Duchenne Muscular Dystrophy	Duchenne Muscular Dystrophy
NCT03368742	Microdystrophin Gene Transfer Study in Adolescents and Children With DMD	Duchenne Muscular Dystrophy
NCT03369444	A Factor IX Gene Therapy Study (FIX-GT)	Hemophilia B
NCT03370172	A Study of BAX 888 in Male Adults With Severe Hemophilia A	Hemophilia A
NCT03370913	Single-Arm Study To Evaluate The Efficacy and Safety of Valoctocogene Roxaparvovec in Hemophilia A Patients	Hemophilia A
NCT03375164	Systemic Gene Delivery Clinical Trial for Duchenne Muscular Dystrophy (DMD)	Duchenne Muscular Dystrophy
NCT03381729	Study of Intrathecal Administration of Onasemnogene Abeparvovec-xioi for Spinal Muscular Atrophy	Spinal Muscular Atrophy
NCT03392974	Single-Arm Study To Evaluate The Efficacy and Safety of Valoctocogene Roxaparvovec in Hemophilia A Patients at a Dose of 4E13 vg/kg	Hemophilia A
NCT03445715	ART-I02 in Patients With Rheumatoid Arthritis With Inflamed Wrists	Rheumatoid Arthritis
NCT03461289	Single-Dose Gene Replacement Therapy Clinical Trial for Participants With Spinal Muscular Atrophy Type 1	Spinal Muscular Atrophy
NCT03466463	Gene Therapy for Severe Crigler Najjar Syndrome	Crigler-Najjar Syndrome
NCT03489291	Dose Confirmation Trial of AAV5-hFIXco-Padua	Hemophilia B
NCT03496012	Efficacy and Safety of BIIB111 for the Treatment of Choroideremia	Choroideremia
NCT03505099	Pre-Symptomatic Study of Intravenous Onasemnogene Abeparvovec-xioi in Spinal Muscular Atrophy (SMA) for Patients With Multiple Copies of SMN2	Spinal Muscular Atrophy
NCT03507686	A Safety Study of Retinal Gene Therapy for Choroideremia With Administration of BIIB111	Choroideremia
NCT03517085	Safety and Dose-Finding Study of DTX401 (AAV8G6PC) in Adults With Glycogen Storage Disease Type Ia (GSDIa)	Glycogen Storage Disease
NCT03520712	Gene Therapy Study in Severe Hemophilia A Patients With Antibodies Against AAV5	Hemophilia A
NCT03520751	Phase I/IIa Trial of scAAV1.tMCK.NTF3 for Treatment of CMT1A	Charcot-Marie-Tooth Neuropathy
NCT03533673	AAV2/8-LSPhGAA in Late-Onset Pompe Disease	Pompe Disease

NCT03536143	Topical Beremagene Geperpavec (KB103) Gene Therapy to Restore Functional Collagen VII for the Treatment of Dystrophic Epidermolysis Bullosa	Dystrophic Epidermolysis Bullosa
NCT03562494	VY-AADC02 for Parkinson's Disease With Motor Fluctuations (RESTORE-1)	Parkinson's Disease
NCT03566043	RGX-121 Gene Therapy in Patients With MPS II (Hunter Syndrome)	Mucopolysaccharidosis
NCT03569891	HOPE-B: Trial of AMT-061 in Severe or Moderately Severe Hemophilia B Patients	Hemophilia B
NCT03580083	RGX-111 Gene Therapy in Patients With MPS I	Mucopolysaccharidosis
NCT03588299	Study to Test the Safety and How Well Patients With Severe Hemophilia A Respond to Treatment With BAY 2599023 (DTX 201), a Drug Therapy That Delivers a Healthy Version of the Defective Factor VIII Gene Into the Nucleus of Liver Cells Using an Altered, Non-infectious Virus (AAV) as a "Shuttle".	Hemophilia A
NCT03612869	Study of AAVrh10-h.SGSH Gene Therapy in Patients With Mucopolysaccharidosis Type IIIA (MPS IIIA)	Mucopolysaccharidosis
NCT03634007	Gene Therapy for APOE4 Homozygote of Alzheimer's Disease	Alzheimer’s Disease
NCT03645460	Gene Transfer for ADA-SCID Using an Improved Lentiviral Vector (TYF-ADA)	Adenosine Deaminase Deficiency Severe Combined Immune Deficiency
NCT03734588	Dose-finding Study of SPK-8016 Gene Therapy in Patients With Hemophilia A to Support Evaluation in Individuals With FVIII Inhibitors	Hemophilia A
NCT03748784	ADVM-022 Intravitreal Gene Therapy for Wet AMD	Neovascular Age-related Macular Degeneration
NCT03758404	Gene Therapy for Achromatopsia (CNGA3)	Achromatopsia
NCT03770572	Gene Therapy for Children With CLN3 Batten Disease	Batten Disease
NCT03818763	Gene Therapy Trial for Platelet Derived Factor VIII Production in Hemophilia A	Hemophilia A
NCT03837184	Single-Dose Gene Replacement Therapy Using for Patients With Spinal Muscular Atrophy Type 1 With One or Two SMN2 Copies	Spinal Muscular Atrophy
NCT03846193	FocuS: First in Human Study to Evaluate the Safety and Efficacy of GT005 Administered in Subjects With Dry AMD	Dry Age-related Macular Degeneration
NCT03882437	Gene Therapy for Male Patients With Danon Disease (DD) Using RP-A501; AAV9.LAMP2B	Danon Disease
NCT03952637	A Phase 1/2 Study of Intravenous Gene Transfer With an AAV9 Vector Expressing Human <=-Galactosidase in Type II GM1 Gangliosidosis	Gangliosidosis
NCT04040049	A Fabry Disease Gene Therapy Study	Fabry Disease
NCT04043104	A Phase 1 Open-Label, Dose Escalation Study to Determine the Optimal Dose, Safety, and Activity of AAV2hAQP1 in Subjects With Radiation-Induced Parotid Gland Hypofunction and Xerostomia	Radiation-Induced Parotid Gland

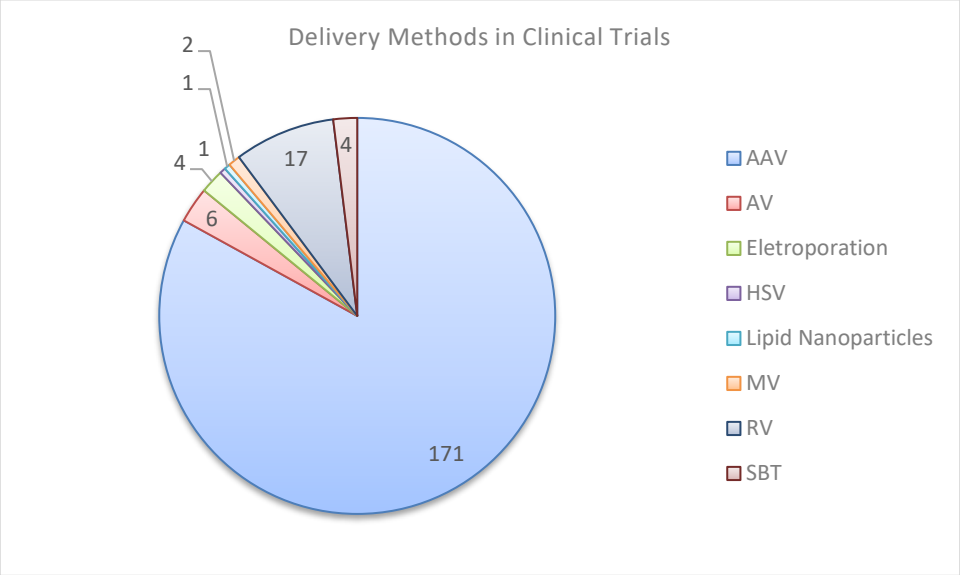
		Hypofunction|Xerostomia Due to Radiotherapy
NCT04046224	Dose-Ranging Study of ST-920, an AAV2/6 Human Alpha Galactosidase A Gene Therapy in Subjects With Fabry Disease	Fabry Disease
NCT04088734	Gene Transfer Study of ABO-102 in Patients With Middle and Advanced Phases of MPS IIIA Disease	Mucopolysaccharidosis
NCT04093349	A Gene Transfer Study for Late-Onset Pompe Disease (RESOLUTE)	Pompe Disease
NCT04105374	Testing the Addition of an Anti-cancer Viral Gene Therapy, Toca 511/Toca FC, to the Usual Treatment (Temozolomide and Radiation Therapy) for Newly Diagnosed Glioblastoma	Anaplastic Astrocytoma|Glioblastoma|Oligodendroglioma|Supratentorial Glioblastoma
NCT04110964	Evaluation of Safety and Tolerability of Libella Gene Therapy for Critical Limb Ischemia: AAV- hTERT	Critical Limb Ischemia
NCT04120493	Safety and Proof-of-Concept (POC) Study With AMT-130 in Adults With Early Manifest Huntington Disease	Huntington's Disease
NCT04127578	Phase 1/2a Clinical Trial of PR001 in Patients With Parkinson's Disease With at Least One GBA1 Mutation (PROPEL)	Parkinson's Disease
NCT04133454	Evaluation of Safety and Tolerability of Libella Gene Therapy for Alzheimer's Disease: AAV- hTERT	Alzheimer's Disease
NCT04133649	Evaluation of Safety and Tolerability of Libella Gene Therapy for the Treatment of Aging: AAV- hTERT	Aging
NCT04135300	Gene Therapy for Chinese Hemophilia B	Hemophilia B
NCT04167540	GDNF Gene Therapy for Parkinson's Disease	Parkinson's Disease
NCT04174105	Gene Transfer Study in Patients With Late Onset Pompe Disease	Pompe Disease
NCT04201405	Gene Therapy With Modified Autologous Hematopoietic Stem Cells for Patients With Mucopolysaccharidosis Type IIIA	Mucopolysaccharidosis
NCT04273269	A Safety and Efficacy Study of LYS-GM101 Gene Therapy in Patients With GM1 Gangliosidosis	Gangliosidosis
NCT04323098	Study to Evaluate the Efficacy and Safety of Valoctocogene Roxaparvovec, With Prophylactic Steroids in Hemophilia A	Hemophilia A
NCT04370054	Study to Evaluate the Efficacy and Safety of PF-07055480 / Giroctocogene Fitelparvovec Gene Therapy in Moderately Severe to Severe Hemophilia A Adults	Hemophilia A
NCT04394286	A Phase 1/2 Study of SHP648, an Adeno-Associated Viral Vector for Gene Transfer in Hemophilia B Subjects	Hemophilia B
NCT04408625	Phase 1/2 Clinical Trial of PR006 in Patients With Frontotemporal Dementia With Progranulin Mutations (FTD-GRN)	Frontotemporal Dementia
NCT04411654	Phase 1/2 Clinical Trial of PR001 in Infants With Type 2 Gaucher Disease (PROVIDE)	Gaucher Disease
NCT04418427	ADVM-022 Intravitreal Gene Therapy for DME	Diabetic Macular Edema | Diabetic Retinopathy
NCT04480567	AAV Gene Therapy Study for Subjects With PKU	Phenylketonuria

NCT04514653	RGX-314 Gene Therapy Administered in the Suprachoroidal Space for Participants With Neovascular Age-Related Macular Degeneration (nAMD)	Neovascular Age-Related Macular Degeneration
NCT04516369	Study of Efficacy and Safety of Voretigene Neparvovec in Japanese Patients With Biallelic RPE65 Mutation-associated Retinal Dystrophy	Retinal Dystrophy Biallelic RPE65 Mutation-associated
NCT04517149	4D-125 in Patients With X-Linked Retinitis Pigmentosa (XLRP)	X-Linked Retinitis Pigmentosa
NCT04519749	An Open-label, Phase 1/2 Trial of Gene Therapy 4D-310 in Adult Males With Fabry Disease	Fabry Disease
NCT04537377	A Phase I/II Study of VTX-801 in Adult Patients With Wilson's Disease	Wilson's Disease
NCT04567550	RGX-314 Gene Therapy Administered in the Suprachoroidal Space for Participants With Diabetic Retinopathy (DR) Without Center Involved-Diabetic Macular Edema (CI-DME)	Diabetic Retinopathy
NCT04611503	PDE6A Gene Therapy for Retinitis Pigmentosa	Retinitis Pigmentosa
NCT04669535	A Dose-escalation and Safety & Efficacy Study of AXO-AAV-GM2 in Tay-Sachs or Sandhoff Disease	Tay-Sachs Disease
NCT04671433	Gene Therapy Trial for the Treatment of X-linked Retinitis Pigmentosa Associated With Variants in the RPGR Gene	X-Linked Retinitis Pigmentosa
NCT04676048	ASC618 Gene Therapy in Hemophilia A Patients	Hemophilia A
NCT04680065	GDNF Gene Therapy for Multiple System Atrophy	Multiple System Atrophy
NCT04684940	Safety, Tolerability, and Efficacy Study of Valoctocogene Roxaparvovec in Hemophilia A With Active or Prior Inhibitors	Hemophilia A
NCT04693598	Gene Transfer Clinical Trial for Krabbe Disease	Krabbe Disease
NCT04703842	Calcium Up-Regulation by Percutaneous Administration of Gene Therapy In Cardiac Disease	Congestive Heart Failure
NCT04704921	Pivotal 1 Study of RGX-314 Gene Therapy in Participants With nAMD	Neovascular Age-related Macular Degeneration
NCT04728841	Gene Therapy for Chinese Hemophilia A	Hemophilia A
NCT04737460	Study for the Treatment for CLN7 Disease	Batten Disease CLN7
NCT04747431	A Study of PBFT02 in Patients With Frontotemporal Dementia and Progranulin Mutations (FTD-GRN)	Frontotemporal Dementia
NCT04783181	A Study of Gene Therapy for Classic Congenital Adrenal Hyperplasia (CAH)	Congenital Adrenal Hyperplasia
NCT04798235	First-in-Human Study of TSHA-101 Gene Therapy for Treatment of Infantile Onset GM2 Gangliosidosis	Gangliosidosis
NCT04832724	RGX-314 Gene Therapy Pharmacodynamic Study for Neovascular Age-related Macular Degeneration (nAMD)	Neovascular Age-related Macular Degeneration
NCT04833907	rAAV-Olig001-ASPA Gene Therapy for Treatment of Children With Typical Canavan Disease	Canavan Disease
NCT04850118	A Clinical Trial Evaluating the Safety and Efficacy of a Single Subretinal Injection of AGTC-501 in Participants With X-linked Retinitis Pigmentosa Caused by RPGR Mutations	X-Linked Retinitis Pigmentosa

NCT04875754	A Study Evaluating the Safety, Tolerability, and Range of Biologically Active Doses of ICM-203 in Mild to Moderate Knee Osteoarthritis	Osteoarthritis
NCT04884815	Clinical Study of UX701 AAV-Mediated Gene Transfer for the Treatment of Wilson Disease	Wilson Disease
NCT04919473	Dose-Escalation Study to Evaluate the Safety and Tolerability of Intravitreal vMCO-I in Patients With Advanced Retinitis Pigmentosa	Retinitis Pigmentosa
NCT04945772	Efficacy and Safety of vMCO-010 Optogenetic Therapy in Adults With Retinitis Pigmentosa [RESTORE]	Retinitis Pigmentosa
NCT02500849	Safety Study of Zinc Finger Nuclease CCR5-modified Hematopoietic Stem/Progenitor Cells in HIV-1 Infected Patients	Human Immunodeficiency Virus 1
NCT04925206	A Safety and Efficacy Study Evaluating ET-01 in Subjects With Transfusion Dependent beta-Thalassaemia	Transfusion Dependent Beta-Thalassaemia
NCT04819841	Gene Correction in Autologous CD34+ Hematopoietic Stem Cells (HbS to HbA) to Treat Severe Sickle Cell Disease	Sickle Cell Disease
NCT04774536	Transplantation of Clustered Regularly Interspaced Short Palindromic Repeats Modified Hematopoietic Progenitor Stem Cells (CRISPR_SCD001) in Patients With Severe Sickle Cell Disease	Sickle Cell Disease
NCT04601051	Study to Evaluate Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of NTLA-2001 in Patients With Hereditary Transthyretin Amyloidosis With Polyneuropathy (ATTRv-PN)	Hereditary Transthyretin Amyloidosis
NCT03872479	Single Ascending Dose Study in Participants With LCA10	Leber Congenital Amaurosis
NCT03745287	A Safety and Efficacy Study Evaluating CTX001 in Subjects With Severe Sickle Cell Disease	Sickle Cell Disease
NCT03655678	A Safety and Efficacy Study Evaluating CTX001 in Subjects With Transfusion-Dependent Beta-Thalassemia	Beta-Thalassemia

Table 1 – List of included clinical trials (CT) including number of clinical trial (NCT), title and target disease.

By analyzing the data collected, it was clear that AAV vectors remain the most widely used delivery systems, corresponding to 83% of all delivery methods used in the included studies (graphic 1). However, non-viral methods are making their way to clinical trials, and in this search a few studies used electroporation, “sleeping beauty transposon” (SBT) vectors and lipid nanoparticles (table 2), representing a total of approximately 4% of the delivery methods (graphic 1).



Graphic 1: Distribution of delivery methods in clinical trials.

Besides delivery methods, the use of nuclease-based editing technologies was also analyzed. These techniques were used in 5,3% of clinical trials, with a predominance of CRISPR/Cas systems. Table 2 shows the list of nuclease-based gene therapy clinical trials.

NCT	*Title*	*Nuclease-based Technique*	*Delivery Method*
NCT02695160	Ascending Dose Study of Genome Editing by Zinc Finger Nuclease Therapeutic SB-FIX in Subjects With Severe Hemophilia B	ZFNs	SBT vector
NCT02702115	Ascending Dose Study of Genome Editing by the Zinc Finger Nuclease (ZFN) Therapeutic SB-318 in Subjects With MPS I	ZFNs	SBT vector
NCT02500849	Safety Study of Zinc Finger Nuclease CCR5-modified Hematopoietic Stem/Progenitor Cells in HIV-1 Infected Patients	ZFNs	SBT vector
NCT03041324	Ascending Dose Study of Genome Editing by the Zinc Finger Nuclease (ZFN) Therapeutic SB-913 in Subjects With MPS II	ZFNs	SBT vector
NCT03745287	A Safety and Efficacy Study Evaluating CTX001 in Subjects With Severe Sickle Cell Disease	CRISPR	Electroporation
NCT03872479	Single Ascending Dose Study in Participants With LCA10	CRISPR	AAV vector
NCT04601051	Study to Evaluate Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of NTLA-2001 in Patients With Hereditary Transthyretin Amyloidosis With Polyneuropathy (ATTRv-PN)	CRISPR	Lipid Nanoparticles
NCT04774536	Transplantation of Clustered Regularly Interspaced Short Palindromic Repeats Modified Hematopoietic Progenitor Stem Cells (CRISPR_SCD001) in Patients With Severe Sickle Cell Disease	CRISPR	Electroporation
NCT04819841	Gene Correction in Autologous CD34+ Hematopoietic Stem Cells (HbS to HbA) to Treat Severe Sickle Cell Disease	CRISPR	AAV vector
NCT04925206	A Safety and Efficacy Study Evaluating ET-01 in Subjects With Transfusion Dependent Beta-Thalassaemia	CRISPR	Electroporation
NCT03655678	A Safety and Efficacy Study Evaluating CTX001 in Subjects With Transfusion-Dependent Beta-Thalassemia	CRISPR	Electroporation

Table 2: Clinical trials using nuclease-based editing and respective delivery methods used.

A variety of diseases are being targeted by gene therapy (table 3), when grouped in disease categories (table 4), inherited retinopathies, hemophilia, lysosomal storage diseases, and neurodegenerative diseases are the most represented in clinical trials.

Target Disease	*No CT*
Achromatopsia	4
Aging	1
AIDS-related malignancies	4
Alpha-1 Antitrypsin Deficiency	3
Alzheimer's Disease	3
Aromatic L-amino Acid Decarboxylase Deficiency	3
Batten Disease	6
Becker Muscular Dystrophy	1
Beta-Thalassemia	2
Bladder Cancer	3
Canavan Disease	1
Cardiomyopathies	5
Charcot-Marie-Tooth Neuropathy	1
Choroideremia	8
Congenital Adrenal Hyperplasia	1
Crigler-Najjar Syndrome	2
Danon Disease	1
Diabetic Macular Edema and Retinopathy	1
Diabetic Retinopathy	1
Duchenne Muscular Dystrophy	7
Dysferlinopathy	1
Dystrophic Epidermolysis Bullosa	1
Fabry Disease	3
Frontotemporal Dementia	2
Gangliosidosis	4
Gaucher Disease	1
Gierke Disease	1
Granulomatous Disease	1
Hemophilia A & B	30
Hereditary Transthyretin Amyloidosis	1
Homozygous Familial Hypercholesterolemia	1
Human Immunodeficiency Virus	3
Huntington's Disease	1
Krabbe Disease	1
Leber Congenital Amaurosis	9
Leber's Hereditary Optic Neuropathy	4
Limb Ischemia	1

Lung Cancer	1
Macular Degeneration	8
Metachromatic Leukodystrophy	1
Mucopolysaccharidosis	11
Multiple System Atrophy	1
Myeloma	1
Myotubular Myopathy	1
Neurological Cancer	2
Ornithine Transcarbamylase Deficiency	1
Osteoarthritis	3
Ovarian/Primary Peritoneal Cancer	1
Parkinson's Disease	9
Parotid Gland Hypofunction	1
Phenylketonuria	1
Pompe Disease	5
Prostate Cancer	1
Retinitis Pigmentosa	13
Retinoschisis	2
Rheumatoid Arthritis	1
Salivary Hypofunction	1
Severe Combined Immunodeficiencies	5
Sickle Cell Disease	5
Spinal Muscular Atrophy	6
Wilson's Disease	2

Table 3: Target diseases and number of clinical trials per disease.

Disease Group	*No CT*
Inherited Retinopathies	40
Hemophilia A & B	30
Lysosomal Storage Diseases	26
Neurodegenerative Diseases	26
Others	19
Muscular Dystrophies	14
Other Neurometabolic Diseases	14
Cancer	9
Hematopoietic Disorders	8
HIV and AIDS-related malignancies	7
Non-inherited Cardiomyopathies	5
Severe Combined Immunodeficiencies	5
Diabetic Macular Edema and Retinopathy	2
Neuromuscular Disorder	1

Table 4: Categorization in groups of diseases and respective representation in clinical trials

On the following pages, a table containing a register of worldwide available treatment is presented, according to the gene therapy definition given in this review (table 5).

Names	*Proper names*	*Indications*	*Companies*	*Approved dates*	*Administrations*	*Regions*	*Fields*	*Vectors*	*Approach*	*Price*
Zynteglo	Autologous $CD34^+$ cells encoding β^{A-T87Q}-globin gene	β-thalassemia	Bluebird Bio	2019.06.03	EMA	EU	Orphan diseases	Lentiviral-β-globin	ex vivo	~$1.8 million/patient
Zolgensma	Onasemnogene Abeparvovec-xioi	Spinal muscular atrophy	AveXis	2019.05.24	FDA	U.S.	Orphan diseases	rAAV9-SMN1	in vivo	~$2.125 million/patient
Collategene	Beperminogene Perplasmid	Critical Limb Ischemia	AnGes MG	2019.03.26	MHLW	Japan	Cardiovascular diseases	Plasmid-HGF	in vivo	N/A
Luxturna	Voretigene Neparvovec-rzl	Biallelic RPE65 mutation-associated retinal dystrophy	Spark Therapeutics	2017.12.19 2018.11.23	FDA EMA	U.S. EU	Orphan diseases	rAAV2-PRE65	in vivo	~$425,000/patient (US;Saudi Arabia) ~$746,000/patient (EU)
Invossa	TissueGene-C	Knee osteoarthritis	Kolon Life Science	2017.07.12	MFSD	Korea	Osteoarthritis	Retroviral-TGF-β1 overexpressing transforming growth factor β1 (TGF β1)	ex vivo	~$4400–7000/patient

Strimvelis	Autologous $CD34^+$ cells transduced to express ADA	ADA-SCID	GlaxoSmith Klinea	2016.05.25	EMA	EU	Orphan diseases	Retroviral-ADA	ex vivo	~$648,000/ patient
Imlygic	Talimogene Laherparepvec	Melanoma	Amgen	2015.10.27 2015.12.16	FDA EMA	U.S. EU	Cancer	HSV1-GM-CSF GM-CSF agonist	in vivo	~$65,000/patient (EU/USA)
Glybera ***Withdrawn***	Alipogene Tiparvovec	Familial lipoprotein lipase deficiency and pancreatitis attacks	UniQure	2012.10.24	EMA	EU	Orphan disease	rAAV1-LPL vector delivers a normal LPL gene	in vivo	~$1.2 million/ patient
Neovasculgen	Cambiogenplasmid	Atherosclerotic peripheral arterial disease, including critical limb ischemia	Human Stem Cell Institute	2011.12.07	Ministry of Healthcare	Russia	Cardiovascular diseases	Plasmid-VEGF in situ (non-viral) delivery, administration of the desired genetic material directly into the target cells or tissue (intramuscular injection)]	in vivo	Less than $50/dose

Rexin-G	Mx-dnG1	Soft tissue sarcoma, osteosarcoma and pancreatic cancer	Epeius Biotechnolgies	2007.12.17	BFADb	Philippines	Orphan diseases	Retroviral vector - dnG1 bearing a cytocidal cyclin G1 construct	in vivo	N/A
Oncorine	Recombinant human adenovirus type 5 injection	Nasopharyngeal cancer	Shanghai Sunway Biotech	2005.11.04	SFDAc	China	Cancer	Genetically modified type 5 adenovirus that selectively replicates on tumor cells with dysfunctional p53 genes	in vivo	~$550/dose
Gendicine	Recombinant human p53 adenovirus particle	Head and neck cancer	Shenzhen SiBiono GeneTech	2003.10.16	SFDAc	China	Cancer	Adenovirus type 5-p53	in vivo	~585$/dose

Table 5: Available gene therapy products worldwide, within the definition of gene therapy proposed on this review. This table was obtained by merging the data contained in *Ma CC et al. (2020) The approved gene therapy drugs worldwide: from 1998 to 2019* and *Alhakamy et al. (2021) The era of gene therapy: From preclinical development to clinical application.*[102,103]

4. DISCUSSION

4.1. Current Clinical Studies and Emerging Issues

In this work, we could realize the importance that AAV vectors still represent for gene therapy. However, new delivery methods and editing techniques are making their way into clinical trials. Although viral vectors have unmatched potential, gene therapies can now complement their role with emerging technologies. It is possible to observe that when available gene therapies use viral vectors, these are generally delivered *in vivo*, and approved for orphan diseases or cancer. In fact, the most differentiating fact between viral and non-viral methods is *in vivo* application. Improvements in non-viral methods to optimize *in vivo* delivery may change the paradigm for delivery techniques.

AAV Vectors

Most clinical trials regarding gene therapies are using AAVs as vectors. AAV vectors use various serotypes and have the ability to infect different tissues, including non-dividing cells. Although AAV vectors are the least immunogenic, viral immunogenicity is always a concern when using viral vectors. Apart from their advantages, these systems have low cargo capacity, and a few strategies, referred previously, were developed to overcome this issue. The development of dual hybrid AAV vectors represents the ultimate effort to optimize these systems. Essentially, the expression cassette is divided between two vectors, one contains the portion encoding the promoter, the 5'-end of the target sequence, the splicing signal, and a bridging sequence, while the other contains the same bridging sequence, a splicing acceptor signal, the 3'-end of the target sequence, and a polyA signal.[104] Since AAV genome undergoes concatemerization in host cells, reconstitution can be accomplished by ITR-mediated concatamerizaton and by bridging DNA sequences through homologous recombination.[104,105]

Another issue with AAV vectors in the increased loss of episomal vector in dividing cells. This made the liver a broadly used target for gene editing, besides being the target of several uncurable monogenic diseases, it is a greatly vascularized organ with a high

number of quiescent cells, and AAV-vectors have an innate capsid hepatic tropism.[106] Along with the liver, AAV vectors are widely used for inherited retinopathies. The eye is a prime target, since it is physically easy to assess, has a blood-retinal barrier that encloses an immune-privileged location and is a small, constrained environment, hindering the need for high doses of vector. [105]

Another approach that has been developed towards AAV vectors is their use in vaccination or the permanent elimination of viral DNA from tissues or cells where sustained viral infection lingers, which depends on the targeted and specific delivery of the therapeutic cargo.[107] Capsids rational design is an area of constant development, useful to all applications, and several strategies, previously referred, provided the ability of choosing vector tropism, avoid off-target entrapment, and lessened immunogenic side effects.[107] However, vector toxicity is not fully under control, dose-dependent side-effects, such as complement activation, cytopenia and severe hepatotoxicity have been a concern over the last decades.[108] Along with capsids development, immunomodulation protocols, improved production systems to avoid contaminants, and removal of pathogen associated molecular patterns (PAMPs) from vector genomes, can all help decrease the risk of such events.[108] Furthermore, although neutralizing antibodies are specific to individual capsids, memory T-cell recognize epitopes, common to different serotypes.[105]

Increasingly variability between gene delivery and gene editing combinations can open a few more possibilities to this field. For instance, an AAV vector can be used to deliver a repair template, while a Cas9 is delivered through a non-viral method. The gradual accumulated knowledge and the recent escalation in the number of gene therapy clinical trials may hold more answers in the following years.

CRISPR/Cas Systems

Analysis of results reveal that although CRISPR/Cas systems are a cutting edge technology for genome editing, it doesn't translate yet in a high number of clinical trials. Since the theme of this review focus on recent advances, it was considered pertinent to include in this discussion cutting-edge developments regarding these systems. In fact, some aspects are still under investigation to find methods with the least off-target

effects, which could be destructive, and prevent other unwanted effects, such as unrestrained immunogenic responses. Also, due to the size and molecular complexity of these systems, efficient delivery strategies are still being developed. Many issues regarding CRISPR/Cas systems, including:

- Cas9 directed immunogenicity,
- minimal understanding of the mechanisms causing off-target effects,
- lack of control over the repair pathway choice, and
- delivery related issues.[13]

These are only a few examples. Due to the potential of this technology, a multiplicity of investigations to optimize these systems is ongoing, in order to widen the range of clinical applications. Several advances regarding the development of CRISPR/Cas systems will be referred here and include a brief explanation or definition.

i) ***Repair pathways.*** The type of gene editing largely depends on the repair pathway applied by the cell. Gene knockout or gene silencing are usually the result of a deletion in the target gene, for which the most frequently used repair pathway is NHEJ. When a double-strand cut is made, NHEJ repairs the blunt ends by linking the loose ends and it is the most used repair mechanism in mammalian cells.[12] However, it is an error prone pathway which frequently leads to insertions or deletions, which generally leads to gene silencing.[12] When the goal is to perform gene insertion or gene editing, the HDR pathway is the most reliable choice. This pathway is followed by the cell if a repair template is available at the moment of the DNA break, which is not always the case. To improve the occurrence of HDR, a repair template can be provided artificially.[12] Throughout the years, investigators have been trying to find a way to selectively lead the cell to perform HDR, and uncovered that cold shock increases the occurrence of HDR.[109] However, HDR seems to be active only during S and G2/M phases, limiting its applications.[12] A better understanding of the mechanism behind this fact introduced the notion that the manipulation of the PALB2-BRCA1/CUL3/Keap1 pathway should be attempted to overcome this limitation.[12,110] When microhomologies exist

between both ends, during the alignment of both double-strands, the microhomology-mediated end-joining (MMEJ, or alternative end-joining) pathway uses the existing complementarities to anneal complementary strands, causing deletions in the nearby regions.[12] This repair pathway apparently works during all phases of the cell cycle, widening its applications to different cell types, and shows high efficiency in knock-in gene editing.[111,112] This technique is under investigation to achieve precise integration into target chromosomes (PITCh).[113] Although NHEJ is usually used to achieve gene knockout, it can also be used to perform gene knock-in through homology-independent targeted integration (HITI), which consists of cleaving the target site on host DNA, while providing a repair template, with two correspondent mimetizing CRISPR target sites flanking the transgene.[12,114] The generation of blunt ends leads to NHEJ, linking the transgene to the hosts' DNA, however, the direction in which integration occurs is unpredictable and off-target effects may be caused by stimulation of transgene integration elsewhere.[12,114]

ii) ***Base Editing.*** Another recent approach to correct monogenic diseases, or to modulate gene expression by introducing termination sequences, is the use of cleavage independent editing techniques. Base editing is a system of base conversion to replace cytidine with uridine, resorting to cytidine deaminases.[12,115] This alteration is then corrected during DNA duplication or by repair mechanisms, such as base excision repair or mismatch repair, and uridine is converted to thymidine.[12] By combining a CRISPR/Cas9 system (engineered to have no cleavage ability) with cytidine deaminases, targeted base editing can be triggered.[116,117] Converting adenosine into guanine is also possible if adenosine deaminases are used instead.[118]

iii) ***Prime editing.*** This approach relies on the fusion of a Cas9 nickase with a reverse transcriptase, carrying a guide RNA which specifies the target site and encodes the desired edit.[119] This technique allows insertions, deletions, and every possible combination of nucleotides, without requiring double-strand breaks or donor DNA templates, which reduces oof-target effects.[119]

iv) ***Paired nickases.*** A nickase is a version of Cas9 without the domains responsible for cutting the complementary strand of the target sequence, only able to cut one strand of DNA.[120] When two modified Cas9 nickases are used together, paired nickases, two adequate PAM sequences must be found on the adjacent sequences, which is highly unlikely to occur naturally. Furthermore, each guide RNA, from each nickase, is complementary to a different strand of the target sequence, a double-strand break would only occur if both proteins would interact with their own target sequence at a close distance. Furthermore, using this mechanism the cleavage creates longer overhangs instead of blunt ends, improving the control over the alterations made, and individual nicks occur, the HDR will run the repair, limiting off-target mutagenesis.[13,121]

v) ***Other Cas9 variants.*** Engineering techniques have led to the production of enhanced Cas9 proteins with increased specificity: i) high-fidelity Cas9 (Cas9-HF1) with decreased mismatches; ii) enhanced Cas9 (eSpCas9) with reduced off-target effects; iii) evolved Cas9 (evoCas9), high-fidelity with residual off-target activity; iv) hyper-accurate Cas9 (HypaCas9) with wide specificity; v) Cas9 with modified PAM sites (x-Cas9, SpCas9-NG, Cas12).[12,122]

vi) ***Anti-CRISPRs.*** Anti-CRISPRs are naturally occurring proteins that can inhibit CRISPR/Cas9 systems' activity, limiting the time frame of Cas9 expression.[122]

vii) ***Enhanced sgRNA.*** Rational design of sgRNA can lead to a higher specificity, by modifying the length, the structure (hairpin sgRNA) or the chemical composition (partially replacing RNA with DNA), therefore *in silico* development of guide RNAs can lead to optimized systems. [12,122]

Delivery of CRISPR/Cas systems. Apart from the previously discussed delivery methods, a few other methods are emerging to tackle the known limitations in CRISPR/Cas systems delivery. Thiolated DNA-coated gold nanoparticles are being used to deliver CRISPR/Cas components, and consist of a DNA template coupled with thiolated DNA, allowing the ribonucleoprotein complex to be attracted by the DNA molecules on the surface of the nanoparticle, which was then coated with negatively charged silica and a cationic polymer (this is frequent due to the systems' negative charge).[123] The choice of

coating can include light sensitive molecules in order to control the systems activity by optical signals.[123,124]

Baculoviral vectors are also being developed as vectors, helping to attain special control over editing systems by encoding Cas9 and the guide RNA into a DNA plasmid, packaged into the baculoviral vector coupled with magnetic nanoparticles. When a magnetic field is applied at the desired tissue, cargo is delivered. This method seems to avoid immunogenicity reactions, since the product is attracted to the target site and does not maintain high concentration in the serum.[123,125]

Other hybrid systems were used, delivering the guide RNA and the repair template through a viral vector and the Cas9 through a non-viral delivery method.[123] Essentially, delivery technologies are evolving side by side, and over time, the combination of different systems as been achieving increasingly complex delivery methods.

Challenges to overcome. Although many progresses have been made, there are still hurdles that need to be overcome to widen the use of CRISPR/Cas9 systems in clinical trials. For instance, CRISPR/Cas9 efficiency seems to be related to p53 expression: it was shown that cancer cells expressing Cas9 upregulate the p53 pathway, and that this accompanied by increased DNA repair activity. [126] A possible consequence is that Cas9 expression might select for p53-inactivating mutations, which might be a relevant problem. [126] In another study, using yeast cells, it was observed that the formation of r-loops when CRISPR/Cas9 interacts with host DNA might lead to mutagenic effects, causing unwanted on- and off-target effects, which must be excluded or bypassed in order to develop safe gene therapies with CRISPR/Cas systems.[12,127]

Other unwanted effects might be caused by NHEJ and include insertions and deletions at the cleavage site, as these alterations can interfere with splicing and lead to uncontrolled expression or expression of gain-of-function proteins with undesired effects[12]. These mechanisms are only superficially studied and further investigations are needed to understand how these occur and can be modulated to increase CRISPR/Cas9 efficiency and reliability. Further studies on base editing systems revealed off-target effects as well, with a frequency 20 times higher than the spontaneous mutation rates.[12,128]

Overcoming CRISPR/Cas9 unwanted effects largely depends on understanding the molecular mechanisms behind interactions between the guide RNA, the target site, and the off-target mutation prone sites. Although prediction systems to identify off-target effects are valuable, if the editing is applied *in vivo*, reliable and thorough methods need to be developed. When applied *in vivo*, especially systemically, it is even harder to assess the damage, since whole-genome sequencing cannot be performed on every edited cell. Immunogenicity is also still a hurdle, since this system is harnessed from bacteria, anti-Cas9 antibodies and anti-Cas9 specific T cells were identified in humans[129], although immunogenic reactions were not described in immunocompromised patients receiving *ex vivo* SpCas9 edited products.[130] Regulatory T cells (Treg) seem to play an important role in inducing tolerance for transgenes, and immunotherapies could be developed to increase Cas9-specific Treg cells in gene therapy treated patients.[130]

Delivery methods have also been evolving continuously, however, finding methods that combine high safety profiles with efficient transfection and high transgene expression rates, remains a challenge. Although promising techniques are being studied, several are limited to *ex vivo* approaches, and clinical applications fall far behind viral vector's mediated gene therapies.

4.2. Gene Editing in Current Medical Practice: Fabry Disease and Spinal Muscular Atrophy

In order to illustrate the significance of new advancements in the gene therapy area for current medical practice, two diseases were chosen: Fabry Disease (FD) and Spinal Muscular Atrophy (SMA). FD has no approved gene therapy treatments yet, although several clinical trials are ongoing and probably new treatments will emerge soon, so it was chosen as an example. SMA has a recently approved new treatment which is now part of the therapeutic offer in clinical practice, illustrating the pertinence of discussing gene therapy as a current medical tool. Both diseases are illustrative examples of what we can expect from gene therapy at this time. Not surprisingly, these diseases are being

treated with gene replacement therapies and most clinical trials use AAV-based vectors to deliver the therapeutic gene.

Fabry Disease

Fabry disease is an X-linked recessive monogenic disease caused by a loss of function mutation in the GLA gene, resulting in a deficiency of α-galactosidase A. This leads to the accumulation of globotriaosylceramide within lysosomes with deleterious effects in several organs.[131] Multisystemic manifestations of the disease occur, such as chronic pain, angiokeratoma, renal disease, cardiomyopathy, cochleo-vestibular disease and ischemic cerebrovascular disease.[132] Males usually display more severe forms of disease, and life expectancy is reduced in approximately 20 years, for males, and 10 years for females. Currently, enzyme replacement therapy (Replagal and Fabrazyme) is the most advanced available treatment. Therapeutic outcomes depend on the age of therapy initiation, development of antibodies, and the maintenance of therapeutic levels with infusions being administered twice a week.[133]

In our search, 6 clinical trials regarding FD were found (NCT04040049; NCT04046224; NCT04455230; NCT04519749, NCT03454893, NCT02800070). All of the referred trials are phase 1/2. Four studies used AAV-based vectors and two used lentiviral vectors. One of them was a follow up study (NCT04455230) on the administration of the same treatments as another trial (NCT04040049), regarding the treatment FLT190. FLT190 and ST-920 (NCT04046224) are treatments based on AAV vectors with tropism for liver tissue, containing DNA encoding the human αGLA gene for in vivo transduction of hepatocytes.[133] Another treatment based on an attenuated AAV vector, 4D-310, was tested in preclinical studies and revealed it could be transduced in human cardiomyocytes with very high efficiency.[133] Other two clinical trials (NCT03454893, NCT02800070) are based on *ex vivo* gene editing of hematopoietic CD34+ cells resorting to a lentiviral vector encoding a functional α-Gal A, which will be secreted by the hematopoietic cells to the plasma, gaining access to every system.[133] AAV vectors rely on the liver tropism of the selected serotypes, in fact, the liver represents 10–15% of the body's total blood volume, which is useful when the interest is production and secretion of proteins into the bloodstream with sustained efficacy.[106] Lentiviral vectors aim to offer permanent gene expression with a unique infusion. Hopefully, in the following

years, results regarding FD gene therapy products originate new treatments for this disease.

Spinal Muscular Atrophy

Spinal muscular atrophy is a neuromuscular disorder that causes degeneration of the alpha motor neurons from anterior horn cells in the spinal cord, leading to hypotonia and muscular weakness.[134] This is an autosomal recessive inherited disease caused by a loss of function mutation in the SMN1 gene, which encodes the motor neuron SMN1 protein. This protein is involved in a series of cellular processes such as assembly of the spliceosome, production of ribonucleoproteins, mRNA trafficking and local translation, cytoskeletal dynamics, endocytosis, autophagy, mitochondria and bioenergetic pathways, and ubiquitin–proteasome system regulation.[135] In humans, there is another gene that encodes this protein, the SMA2 gene and although it mainly produces non-functioning mRNAs, a low percentage are functional SMN2 proteins. Therefore, it can modulate the severity of the disease, originating various phenotypes.[134] SMA manifests through hypotonia, muscular weakness that is usually proximal, and muscular atrophy, in severe cases, bulbar and respiratory muscles are also affected.[134] Understanding the genetic basis of the disease led to development of a few treatments and one of them is a FDA-approved gene therapy product called Zolgensma, which was aproved in May 2019.[134,136]

Zolgensma uses an AAV9-based vector that crosses the blood-brain barrier and delivers a functional copy of the SMN1 gene to the motor neuron cells in the central nervous system with only a single injection. However, it is still unknown how long the therapeutic gene will remain functional in motoneurons, producing the transgenic SMN1 protein.[136,137] Patients treated with Zolgensma in the START study high-dose cohort showed improvements in motor milestones and motor function, and better outcomes were achieved in patients treated at an earlier age.[134,136] A phase 3 clinical trial is being conducted (SPRINT) to access efficacy and safety of gene replacement treatment in pre-symptomatic individuals with 2-3 copies of the SMN2 gene.[134] Until the last update, patients were reaching motor milestones and not requiring mechanical ventilation, liver toxicity was observed, but no other side effects are described.[134]

This achievement shows the importance of gene therapy in current medical practice, and how investing in this field of study can help create innovative treatments for uncurable diseases.

4.3. CRISPR/Cas Systems for Gene Editing in Human Embryos

The development of CRISPR/Cas9 editing systems raised also ethical concerns, particularly when it was applied to develop methods to directly edit the genome of human embryos. Early experiments used clinically discarded embryos and aimed to evaluate the potential of CRISPR/Cas systems for clinical application, by inducing DSB and often adding editing templates, as well as achieving a better understanding of early embryogenesis.[138–143] The main goals of these initial studies in human embryos were to identify on and off-target effects, understand the implications of gene editing in embryo development, evaluate the efficacy level and, finally, to observe mosaicism rates.[144] Many experiments used non-viable tripronuclear embryos, due to ethical concerns, although this complicates the analysis of results, since it is possible that repair pathways are dysfunctional in this type on embryos.[138,142] In general, studies regarding gene editing with CRISPR/Cas systems in human embryos showed low repair efficiency, high rates of mosaicism, large deletions and rearrangement events, and on-target mutations, such as loss of heterozygosity and indels.[145] Mosaicism was observed throughout different editing strategies, and it was associated with the inappropriate timing of editing in relation with cellular division processes.[145] Strategies relying in gene conversion triggered by DSB, which use homologous chromosomes as repair templates instead of an exogenous template, are also under investigation.[145] A preference for interhomolog repair was detected, disabling correction of homozygous mutations when wild-type alleles are not available. Therefore, in-depth studies of early developmental processes and DNA repair pathways in embryos are crucial to understand how editing techniques can be improved.

Since germline gene editing is still a recent area of study, and most mechanisms involving editing platforms are still unknown, the uncovering of a gene editing experiment in human embryos, that were further implanted and born, shocked the scientific community.[146] The man who performed such experiments claims that he could generate human embryos with artificial C-C chemokine receptor type5 (CCR5) mutations, aimed at decreasing susceptibility to HIV.[147] The CCR5 gene encodes a receptor expressed in white blood cells that is used by HIV to achieve cellular infection. A naturally occurring mutation in this gene was observed in some populations in Europe, conferring resistance to HIV or a more favorable disease progression, although infection may still occur.[147] In one of the gene edited babies, sequencing showed one allele was wild-type, and the other contained an in-frame deletion (−15bp). The other baby showed two mutated alleles in all sequencing reads.[147] Official published data is not available to scrutinize the process in depth. However, gene editing human embryos for the sake of an experiment, without even requiring a genetic correction for a disease-causing mutation, risking the known off-target and on-target mutations (such as indels, translocations, rearrangements, large deletions, chromosome truncations, and loss of heterozygosity), that could have unpredictable and devastating consequences, is an outrageous and irresponsible behavior. Furthermore, the performed editing is not proved to be an effective way to achieve HIV immunity, and even if it was, it would only work with certain strains of the virus.

The majority of the scientific community demanded regulatory policies and legislation. China and Japan have already developed regulations, and the World Health Organization put together an advisory committee to globally contribute for regulation and registry of human genome editing experiments.[144] China considers this type of actions as criminal acts and the man responsible for this experiment was arrested.

4.4. Final Considerations

Gene therapy is becoming an important contribution to modern medicine. Due to recent advances in the field, human genome editing techniques have been optimized enough to be used in clinical trials. However, disease mechanisms aren't always deeply understood, and this can bring unexpected results when these therapies are applied. Therefore, the contribution of gene editing techniques for the study of these mechanisms, producing genetically modified animal models, will probably be the lever to a better understanding of pathological processes.

There are several issues that need to be overcome to achieve methodical, effective and precise genome edition. One of the most important steps in limiting error prone genome editing is to understand the mechanisms responsible for off-target and on-target unwanted in depth. The use of human germline cells to study detection techniques and prone off-target sites would be useful to develop improved techniques with high precision mutagenesis detection. At this moment, germline gene editing is the most ambitious goal of gene therapy, which raises ethical concerns.

Licensing policies to the laboratories that work with this type of technologies must be implemented and followed worldwide. With the rapid development of gene editing techniques, it is urgent to discuss this matter as an emerging problem. Conjoined efforts between ethicists, scientists, governments, legal experts and doctors should help create legislation and licensing policies regarding this topic, that should be adopted by governments worldwide. Licensing policies could also be useful to identify gene therapy products more easily. The widening of this scientific field recalls for a review on the definition of gene therapy, and its differentiation from gene editing tools applied in the development of other therapies.

A limitation that emerged during the preparation of this review was the poor design of the database used to search for ongoing clinical trials (https://ClinicalTrials.gov/), as the information there is mostly incomplete. For instance, the level of redundancy between different parts of the same clinical trial is not identified, the description of the methodology (vectors for example) is mostly absent, the trial results are not updated, and the resulting publications with the methods and clinical results are not usually

displayed. These limitations makes it very difficult to systematically identify all the CTs in the area of gene therapy and to critically evaluate the results of this emerging medical technology. Clearly, the existing platforms need to be dramatically improved to facilitate the sharing of knowledge and a critical evaluation of the field.

Gene editing is becoming a therapeutic tool for doctors around the world. The ultimate goal within this field of expertise would be a personalized medicine, not in the near future, but apparently not so far ahead to.

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