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REVIEW PAPER

# CRISPR-Cas9 gene editing as a novel therapy against cystic fibrosis

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## ABSTRACT

Cystic fibrosis is a genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which damages organs such as the lungs and pancreas, predisposing to infections and chronic inflammation. While traditional treatments improve symptoms and quality of life through respiratory therapy, antibiotics, and nutritional support, they do not offer a cure. In this context, gene editing with clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 has emerged as a promising alternative, capable of correcting specific mutations with high precision. Preclinical studies, both *in vitro* and in animal models, have demonstrated its efficacy in restoring CFTR gene function. However, clinical trials are not yet available. Likewise, significant challenges related to specificity and the risk of off-target effects exist, hindering its application in humans. Therefore, it is vital to conduct further studies and establish robust regulatory frameworks to ensure safe, effective, and responsible utilization in the future.

**Keywords:** - *genetic disease, cystic fibrosis, CFTR gene, gene therapy, CRISPR-Cas9*

## INTRODUCTION

Genetic diseases represent a significant health problem for humans, as it is estimated that there are more than 10,000 different conditions affecting approximately 6 to 8 % of the world's population. Their symptoms are highly diverse, with significant clinical or phenotypic variability. Furthermore, they can manifest at any stage of life, [1] and, being hereditary, they are passed down through generations, increasing their complexity and raising concerns. [2] Since the end of the 20th century, advances in decoding deoxyribonucleic acid (DNA) and a deeper understanding of the pathophysiology of genetic affectations have enabled the continuous and growing development of targeted therapies. Strategies have focused on the factors that trigger these illnesses. [1] Human genome sequencing has generated significant advances, enhancing the analysis capacity for clinical purposes. Currently, it is possible to sequence thousands of genomes per year, allowing the monitoring of an individual's transition from health to sickness. Based on this knowledge, multiple projects have been established seeking to manufacture diagnostic tests for at least 6,000 diseases and to find around 200 new treatments. [3] One of these pathologies is cystic fibrosis, which is more common among people of Caucasian descent and occurs in approximately one in every 2,000 to 5,000 births. It is classified as an autosomal recessive genetic disorder, meaning that both parents must contribute a pathogenic allele to their child for the condition to manifest. [4] It is caused

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by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR). [5] The CFTR gene encodes a chloride channel essential for the balance between ion and water secretion and absorption in epithelial tissues, so patients experience multi-organ dysfunction. Mucus thickens in various organs, leading to chronic lung infections and decreased lung function [6] due to inhibition of mucociliary clearance and mucosal obstruction, with consequent airway involvement. [5] It also impairs gastrointestinal function and provokes pancreatic insufficiency. [4] Current clinical management comprises diagnostic tests, regular checkups, and symptomatic treatment ranging from lifestyle changes (exercise and diet) to respiratory therapy and antibiotic administration. The goal is to maintain adequate physical and psychosocial improvement, in line with lung function and structure, to avoid complications related to symptom exacerbation. The need for multidisciplinary treatment affects the patient's quality of life and his family, and poses a challenge for healthcare facilities that provide rehabilitation programs. [7] However, due to its complexity and the genetic variability among individuals, current therapeutic options do not constitute a definitive cure. For this reason, gene editing, through the action of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9, has emerged as a potential cure for monogenic diseases, [8] whose clinical manifestations are caused by spontaneous mutations affecting a single gene, leading to changes in the sequence of the protein that the gene encodes, as is the case with cystic fibrosis. [9]

CRISPR-Cas9 is a highly effective gene-editing tool used in the scientific community. Modifying the nucleotide sequence of a single guide ribonucleic acid (sgRNA) allows for the precise targeting of almost any desired genomic locus [8] (a continuous segment of the genome that encodes for a protein with a specific function) [10, 11] to correct mutations that cause disorders or to silence genes associated with their occurrence. [8] This gene therapy has already caused a stir due to challenges such as off-target effects, efficient gene delivery, and ethical concerns, particularly the possibility of unintended consequences. Therefore, challenges lie ahead in ensuring its durability, safety, and efficacy in humans. [12] Thus, the objective of this article is to examine gene editing utilizing CRISPR-Cas9 technology as a therapeutic strategy for cystic fibrosis.

## ANATOMY AND PHYSIOLOGY OF THE LUNGS

The respiratory system anatomy involves a series of structures that allow gas exchange between the environment and the blood. Anatomically, it is divided into the upper airways, including the nose, pharynx, and larynx, and the lower airways, encompassing the trachea, bronchi, bronchioles, alveolar ducts, and alveoli. Functionally, it is described as two zones: the conducting zone for inhaled gases (from the nose to the bronchioles) and the respiratory zone (from the alveolar ducts to the alveoli), where gas exchange occurs. [13] The airway has been divided into functional compartments. These structures include a proximal conducting zone, consisting of the tracheobronchial tree up to generation 16; a transition zone (generations 17 to 19); a respiratory zone (generations 20 to 22); and the alveolar region. [14] The generations correspond to successive divisions of the respiratory tree, with each branch from the trachea (generation 0) to smaller structures numbered sequentially. [13] The lung is the main organ that has two essential, interdependent functions. The first is ventilation-perfusion, which supplies oxygen to the body and removes carbon dioxide produced. Its second role is defense against pathogens, chemicals, and airborne particles. [15] The alveoli are the gas-exchange site, surrounded by type I pneumocytes, the specific exchange site, as well as type II pneumocytes, which produce surfactant, a substance that reduces surface tension at the alveolar air-liquid interface and prevents the collapse of the terminal bronchioles and the alveolar zone. [14] The relationship between the lungs and cystic fibrosis is central to the pathophysiology, since the primary alteration in epithelial ion transport leads to chronic obstructive pulmonary disease. [16] This situation favors recurrent infections, persistent inflammation, and progressive structural damage. [17]

## PATHOPHYSIOLOGY OF CYSTIC FIBROSIS

When the condition was described by Dr. Dorothy Anderson in 1938, most patients died within their first year of life. Today, the proportion of adult patients exceeds that of children in developed countries, and the estimated median life expectancy is 50 years. This change is primarily due to the discovery of

the CFTR gene, which triggers it. Since then, epidemiological changes have occurred through the implementation of genetically informed health policies for early diagnosis and prevention within the population, and through the emergence of modulatory therapies targeting this gene. [18] Cystic fibrosis is a monogenic disorder transmitted in an autosomal recessive pattern. It arises from a mutation in the gene encoding CFTR, located on chromosome 7q31.2. [19] Not all mutations produce its phenotypic effect. Approximately 2,000 CFTR gene alterations have been identified, of which only 360 are disease-causing. Almost all consist of a change in three or fewer nucleotide base pairs, resulting in frame shifts, splice sites, nonsense mutations, or amino acid substitutions. The implications range from defects in protein synthesis to impaired cell membrane stability. Mutations are classified according to their effects and severity. Classes I, II, and III cause the most severe pathology form and lead to classic cystic fibrosis, while classes IV, V, and VI result in milder manifestations. [20]

CFTR is a cAMP-dependent chloride channel and belongs to the ATP-binding cassette (ABC) protein family. [20] This family of integral membrane macromolecules contains a core structure composed of two half-transporters, two transmembrane domains (TMDs), and two nucleotide-binding domains (NBDs). The binding and hydrolysis of ATP in NBDs in many cells enhance the solute's active transport across membranes. In contrast, others permit them to function as channels or to regulate the activity of interacting proteins. This capacity makes them involved in diverse biological processes related to the transport of substances. CFTR is primarily located in the plasma membrane of epithelial cells, particularly in the apical membranes that line the upper and lower airways, sinuses, parts of the gastrointestinal tract, exocrine pancreatic ducts, and the vas deferens. [16, 20] Pathogenic mutations lead to inadequate and inefficient chloride transport across the epithelia's apical membranes because the channel is no longer present in the membrane, becoming impermeable or exhibiting limited chloride permeability. This scenario promotes alterations in fluid and electrolyte balance and triggers changes in mucus composition, ultimately leading to organ dysfunction and death. [20]

Its most common clinical manifestation is progressive lung disease. Additional manifestations may involve bronchiectasis [20, 21] (irreversible widening of small to medium-sized airways), [22] respiratory tract infections, pancreatic insufficiency, salty sweat (due to increased NaCl excretion), reproductive complications, and intestinal obstruction. [20, 21]

Progressive lung disease is the principal determinant of morbidity and mortality. [23] In the airways, CFTR regulates local pH by allowing chloride and bicarbonate ions flow out of the cell. Additionally, it regulates the epithelial sodium channel (ENaC) activity, which transports sodium into the cell. When present, the pH is up to eight times more acidic because of inadequate secretion of the aforementioned anions into the extracellular space, generating significant variations in osmotic pressure and electroneutrality, leading to excessive sodium and water absorption, with subsequent augmented mucus viscosity and difficulty in its clearance from the airways. [16] Symptoms that trigger complications comprise dyspnea, cough, reflux, weight loss, depression or anxiety, sleep disturbances, fatigue, and general malaise. These symptoms are described as part of the profile that accompanies the progressive development of the illness. [24] Moreover, the persistence of the lung disease facilitates endobronchial bacterial infections, further complicating its course [16] and leading to complications such as hemoptysis and pneumothorax. [25] On the other hand, key signs of its presence are bibasilar inspiratory crackles (a Velcro-like crackle sound auscultated at both lung bases). [26] Similarly, situations of nocturnal oxygen desaturation should be monitored. [27]

## EPIDEMIOLOGY OF CYSTIC FIBROSIS

Cystic fibrosis most frequently affects Caucasians. [16] For a time, it was believed to affect only people of European descent and to be prevalent in Europe, North America, and Australia. Nonetheless, recent studies have shown that it is present, albeit at lower rates, in other regions, including the Middle East, Asia, and Latin America. [28] The belief that it affects almost exclusively high-income countries stems from the fact that these territories have established better systematic registries. [28] National registries are essential for understanding population dynamics, disease progression, and the effectiveness of clinical interventions. In addition, they allow for the comparative evaluation of national and international health centers and systems. [18] Currently, many nations have improved their registries,

providing more information about their global distribution. However, the lack of efficient registries in regions such as the Middle East, Latin America, Africa, and Asia has prevented an accurate global estimate of the number of people with the illness. [18] Therefore, the incidence currently ranges from 1/3,000 to 1/6,000 live births in populations of European descent. In Latin America, the average is approximately 1/10,000 to 1/8,000, ranging from 1/15,000 in Costa Rica to 1/6,100 in Argentina. Other rates are 1/350,000 in Japan, between 1/10,000 and 1/100,000 in the Indian population, and 1/2,560 in Jordan. [29]

Incidence rates in distinct regions depend on various factors. Some of them are demographic changes, implementation of genetically based health policies for prevention within families or populations (prenatal diagnosis, preimplantation genetic diagnosis, family testing, and carrier screening), and cultural attitudes toward genetic testing, prenatal diagnosis, and pregnancy terminations. Due to these factors, a robust national registry is essential for conducting more accurate population analyses. [18]

Survival rates have improved significantly over the years. They are related to factors such as CFTR genotype, initial presentation of the genetic disorder, nutritional status, sputum bacteriology, diabetes, socioeconomic status, pulmonary exacerbations, sex, and follow-up at a specialized center. [30] Besides, low socioeconomic status is associated with food insecurity, air pollution, limited access to rapid diagnosis, and specialized treatments due to their high cost and lack of health insurance coverage. In England and Wales, children from lower socioeconomic groups have been observed to be almost three times more likely to die at a young age than those from higher socioeconomic backgrounds. Similarly, in Latin America, patients with low socioeconomic status exhibit poorer lung function, lower body mass index (BMI), more pulmonary exacerbations, and a higher risk of *Pseudomonas aeruginosa* infection. [30]

## TRADITIONAL TREATMENT OF CYSTIC FIBROSIS

Traditional management considers factors that require special attention: making an early diagnosis, keeping the airways clear of secretions and infections, maintaining optimal nutritional status, and establishing specialized multisystem management. [31] Early diagnosis aims to treat affected individuals at the earliest stage, thereby incrementing patient survival and life expectancy. The immunoreactive trypsinogen (IRT) test implies measuring IRT levels in a drop of dried blood taken from the heel. Elevated IRT levels result from partial or total pancreatic exocrine duct obstruction, which prevents the enzyme from being released into the plasma. The first IRT test is carried out between days 3 and 5 after birth, and again between days 25 and 40. If the second test is positive, genetic testing is made. This assay is done on the same capillary blood sample obtained previously, analyzing the most prevalent variants locally, usually screening for 50 variants of the CFTR gene. [32]

Secondly, maintaining the airways clear of secretions should begin at diagnosis and become part of the patient's daily life. Respiratory physiotherapy helps by applying techniques tailored to age. In children under three years of age, passive chest percussion, blocks, vibrations, and postural drainage are recommended. After that age, forced expiration techniques can be added, encouraging the person to exhale slowly and progressively, moving secretions toward the central airway. [31] Pharmacological agents that promote the clearance of secretions are administered. Options involve dornase alfa (recombinant human DNase), which degrades the DNA that accumulates in the airways of patients with cystic fibrosis, reducing mucus viscosity. Mucosal hydrators, such as hypertonic saline solutions and mannitol, create an osmotic gradient that draws water into the airways and rehydrates airway surface fluid, thereby augmenting mucociliary clearance. [33] Furthermore, keeping the airway free of infection prevents exacerbations and chronic infections that can impair respiratory health and lung function. It is crucial to anticipate the onset of symptoms by performing regular cultures of bronchial or pharyngeal secretions (when expectoration is absent) to initiate antibiotic therapy promptly. [31] *P. aeruginosa* and *Staphylococcus aureus* are two of the most frequently detected bacteria in the lungs of these patients. For their elimination, inhaled antibiotics are preferred, owing to their ability to achieve higher concentrations in the respiratory tract and to generate less systemic toxicity. [34] The mechanisms of action and the microorganisms against which they are used are described in **Table 1**.

**Table 1.** Mechanism of action of antibiotics used against *P. aeruginosa* and *S. aureus* in patients with cystic fibrosis. [34]

| Antibiotic | Mechanism of action | Pathogen |
|---|---|---|
| **Amikacin** | It is a broad-spectrum aminoglycoside that inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit. | *P. aeruginosa* |
| **Aztreonam** | The molecule is a synthetic beta-lactam that disrupts bacterial cell wall synthesis by blocking peptidoglycan crosslinking. | *P. aeruginosa* |
| **Colistin** | It alters the bacterial plasma membrane by interacting with lipopolysaccharides (LPS) in the outer membrane of Gram-negative bacteria, displacing the LPS-stabilizing ions $Mg^{2+}$ and $Ca^{2+}$, thereby disrupting the outer membrane, increasing the permeability of the cell envelope, and causing cell death. | *P. aeruginosa* |
| **Fosfomycin** | It affected the synthesis of the cell wall of Gram-positive and Gram-negative bacteria. | *S. aureus* |
| **Levofloxacin** | It is a fluoroquinolone that inhibits bacterial DNA replication by targeting both DNA gyrase and topoisomerase IV, leading to cell division failure and death. | *P. aeruginosa* |
| **Murepavadin** | It binds to lipopolysaccharide transport protein D (LptD) in Gram-negative bacteria, inhibiting its LPS transport function, disrupting outer membrane assembly, and promoting cell death. | *P. aeruginosa* |
| **Tobramycin** | The antibiotic binds to 30S ribosomal subunits and inhibits bacterial protein synthesis. | *P. aeruginosa* |
| **Vancomycin** | It acts by inhibiting cell wall synthesis in Gram-positive bacteria. | *S. aureus* |

Additionally, optimal nutritional status should be promoted, as there is a close relationship between BMI and lung function. The goal is to maintain a BMI of 22 for women and 23 for men. To achieve these values, periodic assessment and appropriate interventions are necessary. Enzyme replacement therapy is administered in cases of pancreatic insufficiency (80 to 85% of patients) with replacement enzymes before each meal. [31]

Conversely, replacement therapy for fat-soluble vitamins (A, D, E, and K) is recommended, and a high-calorie, high-protein diet should be followed to compensate for increased energy expenditure, along with maintaining bone health and monitoring blood vitamin D levels. Annual glucose tolerance testing is required to prevent the progress of diabetes mellitus, which can result from declines in nutritional status and lung function. Finally, liver function tests should be performed annually. [31]

Finally, care at a specialized center is essential for a multidisciplinary team, facilitating patient management. This management makes it easier to find solutions to the problems presented. [31] It is important to emphasize that the approach described above aims to reduce complications. Still, it does not cure the genetic disorder. Hence, there is a necessity to develop new treatments.

## MUTATIONS IN THE CFTR GENE AND THEIR RELATIONSHIP WITH CYSTIC FIBROSIS

Cystic fibrosis results from mutations in the CFTR gene, [35] leading to an abnormal buildup of thick, sticky mucus that blocks the airways, glands, and digestive tract. [36] They are divided into six classes. Each one can be associated with specific types of gene dysfunction, as shown in.

**Table 2**. Mutations, effects on the CFTR protein, and related examples of dysfunction. [37, 38]

| Class | Molecular mechanism | Effect on the protein | Examples of amino acid modifications |
|---|---|---|---|
| **I** | Nonsense, frameshift, or splice-site mutations, leading to premature termination of the messenger RNA (mRNA) transcripts. | No protein synthesis or translation of shortened, truncated forms. | G542X, R553X, R1162X, W1282X, 621+G>T, 1717-1G>A |
| **II** | Misfolding protein fails to achieve conformational stability in the endoplasmic reticulum. | Premature proteasomal degradation occurs because of inadequate trafficking to the correct cellular location. | G85E, I507del, F508del, N1303K |
| **III** | Diminished channel activity even when ATP levels are adequate, and alterations in nucleotide-binding folds and ATP-binding regions could be present. | Gating channel defect. | S549R, G551D, G1349D |
| **IV** | Macromolecule is produced and transported correctly to the cell surface, but the ion flow rate and channel opening duration are reduced. | Significant reduction in CFTR-dependent chloride transport. | R117H, R334W, D1152H |
| **V** | mRNA stability and CFTR protein maturation are altered. | Reduction of functional CFTR protein. | A455E, 2789+5G>A 3849+10kbC>T |
| **VI** | Substantial plasma membrane instability. | Reduced protein stability at the plasma membrane, augmenting endocytosis and lysosomal degradation. | rPhe508del, Q1411X |

In general, mutations I, II, and III are more severe. Nonetheless, the clinical manifestations by any particular combination can vary. [37] Class I mutations result in a severe reduction or absence of CFTR due to the introduction of premature termination codons from frameshift, nonsense, or splicing mutations. [39] Some of them involve G542X and W1282X. [39, 40] Class II mutations lead to misfolding and premature degradation by the endoplasmic reticulum, decreasing the macromolecules that reach the cell surface. [39] F508del is one example. Class III mutations exhibit a gating defect, [40] as does G551D. [41] Less severe mutations, such as those in class IV, cause defective conduction because the channel openings and flow rates are incorrect. R117H, R334W, and D1152H are in this group. Likewise, class V mutations reduce the amount of functional protein, and class VI mutations decrease its stability. [37, 38] Examples of the latter are A455E [42] and Q1412X, [43] respectively. The significance of some mutations in the CFTR gene is summarized in **Table 3**.

**Table 3**. Review of some mutations in the CFTR gene.

| Mutation | Explanation |
|---|---|
| **A455E [44]** | Alanine (A) replaces glutamate (E) at position 455. |
| **F508del [36]** | Deletion of phenylalanine (F) at position 508. |

| | |
|---|---|
| **G542X [40]** | A stop codon replaces glycine (G) at position 542. |
| **G551D [41]** | Glycine (G) replaces aspartic acid (D) at position 551. |
| **R117H [45]** | Arginine (R) replaces histidine (H) at position 117. |
| **W1282X [46]** | A stop codon replaces tryptophan (W) at position 1282. |
| **Q1412X [43]** | A stop codon replaces glutamine (Q) at position 1412. |
| **3849+10kbC>T [47]** | Cytosine (C) replaces thymine (T) at position 3849, specifically 10 kilobases into intron 19. |

The most frequent mutation worldwide linked to this pathology is the one at position 508, which results in the loss of phenylalanine due to a 3-nucleotide deletion. It is present in approximately 70 % of cases. The protein breaks down shortly after it is produced, before it can reach the cell membrane to transport chloride ions. [36]

Mutations vary considerably in distinct geographic regions. In a study of the Cuban population, after analyzing samples from 252 patients across several areas, the most frequent mutation nationwide, particularly in the central region, was F508del, followed by G542X and R334W in the eastern region. At the same time, R553X was not detected in the latter. Other variants identified were R1162X and 3120+1G>A. These findings showed significant genetic diversity, influenced by the Cuban population's ancestry (European, African, and Native American) and by migration across the country. [48]

Another investigation conducted in Iran found that in 56 individuals, the F508del variant had a frequency of 10.71%, a low value compared to the incidence reported in Europe. The mutations 1677delTA, R344W, S466X, L467F, G542X, 1677delTA, and N1303K were observed, although less frequently. [19]

Meanwhile, an analysis in India was conducted with 120 patients and 55 variants of the CFTR gene. The most common was F508del (27 %), again lower than in European populations. Likewise, significant regional variations in genotype frequencies and clinical manifestations were observed among the regions studied. [49]

## STRATEGIES FOR GENE EDITING

Currently, three gene editing strategies are recognized: transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and CRISPR-Cas9. These tools generate targeted DNA cuts, resulting in genome modification. [50] TALENs comprise a class of proteins that can bind to DNA. They are highly compatible with many functional domains, thus offering diverse applications. TALE proteins were derived from the phytopathogenic bacterial genus *Xanthomonas*. [51] Two DNA-binding domain (DBD) units were fused with the catalytic domain of the restriction endonuclease FokI. The methodology is precise, can be employed in numerous organisms, and can modify nuclear and mitochondrial DNA. [51, 52] Regarding ZNFs, combined with the nuclease domain of the restriction endonuclease FokI, they create double-strand breaks (DSBs) at precisely defined genomic locations. [53] Although this technology requires a complex, less flexible design, it offers advantages such as high specificity and stability, with little risk of off-target effects. [54]

For its part, CRISPR-Cas9 has advantages compared to TALENs and ZFNs. Most Cas9 genome-editing purposes use sgRNAs by fusing the CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) into a single RNA molecule. [55] At the same time, the other two involve cloning and engineering with y more complex proteins, translating into advantages in terms of cost, complexity, and time. [56] Furthermore, multiple sgRNAs can be introduced, and a single Cas9 protein can be sufficient to edit various loci simultaneously, in contrast to the other two, which require a different protein for each target. [57]

## MECHANISM OF ACTION OF CRISPR-CAS9 AND ITS APPLICATION IN CYSTIC FIBROSIS

The CRISPR-Cas9 system was first described in 1987 using *Escherichia coli*. It consisted of an adaptive immunity mechanism in bacteria and archaea to prevent viral infections. [58] This tool provides microbes with RNA-guided adaptive immunity to foreign genetic elements by directing nucleases to bind and cut specific nucleic acid sequences. [59] Subsequently, in 2012, it was adapted for genetic engineering through the bacterium *Streptococcus pyogenes*. [58] The DNA cutting mechanism is guided by RNA-determined sequence recognition. The sgRNA directs Cas9 to the target site, where it creates a DSB that is repaired by endogenous repair pathways, including nonhomologous end joining (NHEJ) and homology-directed repair (HDR) pathways. Because these proteins use RNA-DNA base pairings for DNA recognition, the same protein can be applied to a wide range of DNA sequences by swapping sgRNAs. [60] There are six types of CRISPR-Cas systems, grouped into two classes according to the sequence and structure of the Cas nucleases. Class 1 includes types I, III, and IV, while Class 2 includes types II, V, and VI (**Table 4**).

**Table 4**. Classification and characteristics of the diverse types of CRISPR-Cas systems.

| Class | Type | Characteristics |
|---|---|---|
| 1 | I | A multiprotein complex bound to crRNA, called the Cas complex, for antiviral defense, and the Cas3 endonuclease, recruited after binding to the target by the endonuclease, to cleave foreign DNA. [61] |
| | III | Composed of a Cas10 complex, which has two catalytic domains, the histidine-aspartate (HD) domain, which cleaves single-stranded DNA (ssDNA), and the Palm domain, which synthesizes cyclic oligoadenylates. [62] |
| | IV | Encoded primarily by plasmids and occasionally by prophage genomes. They associate with CRISPR arrays. However, they lack distinctive components, including the highly conserved adaptation module and an effector nuclease. [63] |
| 2 | II | Composed of Cas9, a large multidomain nuclease containing two domains, a RuvC-like (RNase H fold) domain and an HNH (McrA-like fold), responsible for DNA excision. [64] |
| | V | It consists of a single RNA-guided RuvC domain-containing Cas12. [65] |
| | VI | Composed of Cas13, which assembles with crRNA, forming an RNA-directed effector complex. [66] |

Each system achieves nucleic acid recognition and cleavage through distinct molecular mechanisms. Types I and III are not utilized for genetic modification due to their protein complexity. [67] In contrast, the Type II (Cas1, Cas2, Cas4, and Cas9) needs only one protein to perform its function of recognizing and cutting DNA guided by crRNA, making it a simpler and more efficient structure for gene editing. Additionally, the Cas9 protein has been mutated to enhance its specificity, making it the most widely used system. [58, 67]

Some strategies to improve specificity incorporate structure-guided rational engineering of Cas9, directed evolution to identify and apply nuclease variants with greater specificity, the creation of obligate heterodimers using the Cas9 nuclease or fusion proteins such as dCas-FokI, and modification of the sgRNA by truncation or alteration of the scaffold sequence. [68, 69]

Target binding also depends on a protospacer adjacent motif (PAM), consisting of three base pairs, [57, 70, 71] which is located on the non-target strand (NTS) of the DNA, immediately downstream of the target site. When Cas9 recognizes the PAM, it induces the DNA to unwind and allows the crRNA to bind to the target DNA strand, following the 5′ to 3′ direction. Once proper pairing occurs, Cas9 undergoes a conformational change that activates its nuclease domains and initiates DNA cutting. [70]

The DSB break is repaired via two pathways. The first is NHEJ, which does not require a DNA template. [72] This pathway operates throughout the cell cycle, facilitating the processing, synapsis, and ligation of DSB ends through a set of flexible mechanisms. [71] The broken DNA ends are rejoined by different proteins, including DNA ligase, Ku70/80, and DNA-PKcs. Ku70/80 is a heterodimer complex

of 70 and 80 kDa subunits that detects and binds to the broken DNA ends. Subsequently, the DNA-PKcs protein binds to this heterodimer, forming the DNA-PK complex. The process is carried out by the Artemis nuclease, which has an extended C-terminal end of 300 amino acids with flexible, low-complexity regions that bind to DNA-PKcs. DNA ligase participates in the repair by joining a scaffold assembled from XLF and XRCC4 protein filaments, which act as support structures during the joining of non-homologous ends, facilitating ligation. [72] While it does not require a repair template, it is of limited accuracy and prone to errors because of the high likelihood of DNA insertion or deletion mutations. [67]

The second pathway occurs via HDR. The mechanism takes place in several steps. First, the DNA on each side of the DSB is resected in the 5' to 3' direction, forming 3' overhangs at each end. These overhangs are stabilized by replication protein A (RPA), which then binds to the Rad51 recombinase, each creating a nucleoprotein strand. Then, a nucleoprotein strand invades a homologous sequence on an intact DNA molecule, which becomes the repair template. This invasion results in a displacement loop, in which the 3' end of the damaged DNA pairs with the complementary strand of the template, displacing the other strand. From there, DNA polymerase extends the strand using the template as a guide. [73] This pathway is less efficient than NHEJ, since NHEJ is active throughout the cell cycle. HDR is only efficient during the S and G2 phases. [73]

Because it can cut specific DNA sequences, the CRISPR-Cas9 system has enormous potential to correct genetic mutations that generate hereditary disorders such as cystic fibrosis. This process can be achieved by a specific guide RNA that recognizes the mutated sequence of the disease-causing gene and corrects the affected locus. [74] Research on other pathologies, such as transfusion-dependent β-thalassemia and sickle cell anemia, has shown results supporting the potential of the therapy. [75, 76] Nevertheless, specificity problems must be considered. Cas9 tolerates up to 3 mismatches between the sgRNA and genomic DNA, leading to off-target effects. [77] This limitation poses the risk that the system will not correctly identify the specific sequence, contributing to unintended genetic modifications and changes in the genome, as seen in other animal studies, where these mutations occurred in 29 % of the mice. [78]

## MAIN RESULTS OF PRECLINICAL AND CLINICAL STUDIES ON GENE EDITING USING CRISPR-CAS9 TECHNOLOGY FOR THE TREATMENT OF CYSTIC FIBROSIS

In relation with the principal results in preclinical investigations on gene editing employing CRISPR-Cas9 technology for the treatment of cystic fibrosis, *in vitro* studies have been developed through cell models with heterologous pathways, which are interconnected series of biochemical reactions that occur in a host organism after the introduction of foreign genes, [79] immortalized epithelial cells, which have been manipulated to proliferate indefinitely and can be cultured for long periods, [80] induced pluripotent stem cells (iPSCs) from patients, which comprise stem cells produced from somatic cells throughout the co-expression of defined factors associated with pluripotency, [81] and intestinal or pulmonary organoids, which are miniaturized, three-dimensional structures that recapitulate cellular tissues. [82] Likewise, *in vivo* models are available, employing animal models with CFTR mutations. [12]

In the preclinical phase, it is noteworthy that CRISPR-Cas9 has successfully corrected common CFTR mutations (F508del, G542X, W1282X) in cell models, restoring CFTR function *in vitro*. Research indicates F508del corrections of approximately 22 % without selection in iPSCs, [83] meaning no system was designed to select the edited cells specifically.

Similarly, another study utilizing iPSCs derived from patients with a homozygous F508 deletion in the CFTR gene, resulting in defective protein processing to the cell membrane, was conducted. The CRISPR-Cas9 system corrected the mutation by targeting corrective sequences to the endogenous genomic locus, thereby significantly improving correction efficiency. The corrected iPSCs were subsequently differentiated into mature airway epithelial cells, demonstrating the recovery of normal CFTR expression and function. [84]

In one investigation, CRISPR-Cas9 with HDR was applied, as it is a versatile platform for creating precise DNA insertions, deletions, and substitutions at specific sites [85] to correct the F508del mutation directly in intestinal stem cells. A Cas9 plasmid with sgRNA targeting the mutant CFTR locus and a

repair DNA template with the correct sequence was designed. [86] In this case, a genetic correction strategy using homologous recombination (HR) was considered in clonogenic intestinal stem cells derived from biopsies of cystic fibrosis patients homozygous for the F508del/F508del mutation. These stem cells were transfected to correct the mutation. Following transfection, cell clones that had successfully incorporated the CFTR gene correction via HR were selected. These corrected cells were selected to generate intestinal organoids. As a result, the expression of the corrected CFTR allele was fully functional, demonstrated by a relative increase in the total surface area of the organoid to 167 and 177 % in two corrected small intestine organoid clones, and to 180 and 187 % in two corrected large intestinal organoid clones, evidenced by the swelling response, which was comparable to that of healthy organoids. The swelling indicates that CFTR is functioning correctly. [86]

In another assay, human nasal epithelial cells homozygous for the G542X mutation were manipulated to target the adenine base editor 8e (ABE8e), a Cas9 nuclease with adenine deaminase, and sgRNA/enhanced green fluorescent protein plasmids encapsulated in receptor-targeted nanoparticles. The purpose was to transform the stop codon into G542R, a variant that responds to the modulator therapy (VX-445/VX-661/VX-770). The correction was evaluated in differentiated cell cultures under air-liquid interface (ALI) conditions, [87] a tool for recreating in vitro the typical features of the respiratory tract in healthy and diseased states. [88] The gene editing approach achieved an efficiency of 52%, resulting in improved messenger RNA and CFTR protein levels. Thus, editing G542X to G542R with a base editor can rescue its function in human cells. [87]

One investigation consisted of optimized lung-targeting lipid nanoparticles (LNPs) with high lung-targeting efficiency to deliver CRISPR-Cas9 and ABE to lung stem cells in a murine model of cystic fibrosis. The assay focused on the R553X nonsense mutation, a common mutation among patients, which converts adenine to guanine at position 553. This intervention corrected the alteration, returning it to the typical sequence that encodes for a functional protein. The method achieved a genome-editing efficiency of over 70% and sustained gene expression of 80% for more than 660 days. In addition, over 95% of CFTR gene correction was achieved. [89]

As a complement, CRISPR-Cas9 and two adeno-associated viruses (AAVs) were made available from 11 donors carrying various cystic fibrosis-associated mutations. The AAVs were utilized to transport the two halves of CFTR complementary DNA (cDNA) to sequentially insert the complete CFTR cDNA, along with an enrichment tag based on a truncated version of CD19 (tCD19), into upper airway basal stem cells (UABCs) and human bronchial epithelial cells (HBECs). After tCD19 selection, populations with 60 to 80% edited cells were obtained. This research showed that restored CFTR function exceeded 70% compared to controls without the pathology. [90]

Besides, an investigation was conducted using Lung Selective Organ Targeting LNPs (SORT LNPs) to identify efficient delivery vectors for genome editors that could be useful in CRISPR-Cas gene therapy. The aim was to achieve efficient delivery of Cas9 mRNA, sgRNA, and ssDNA with Lung SORT LNPs, enabling homology-directed repair-mediated gene correction in cystic fibrosis models. Treatment with SORT LNPs successfully corrected CFTR mutations in homozygous G542X mice and in human bronchial epithelial cells derived from patients with homozygous F508del mutations, restoring protein expression and chloride transport function. [91]

Electroporation of CRISPR-Cas9 and sgRNA ribonucleoprotein (Cas9 RNP) has also been contemplated to deliver efficient therapy targeting loci harboring important CFTR mutations. [92] Electroporation requires electric fields to create transient pores in the cell membrane, allowing the entry of macromolecules. [93] This study was conducted without the need for viral vectors, drug selection, and reporter enrichment. As a result, efficiencies of 4.8 to 27.2 % were achieved for F508del, G542X, and G551D mutations in iPSCs. The correction restored CFTR function in iPSC-derived proximal lung organoids and in a patient-derived adenocarcinoma cell line, CFPAC-1. [92]

Additionally, vectors have been created to test CFTR gene correction in porcine models, specifically helper-dependent adenoviral (HD-Ad) vectors that deliver CRISPR-Cas9 and a donor template, either a 6 kb LacZ or an 8.7 kb human CFTR expression cassette, into cultured porcine cells. [94] In the case of LacZ, this is the bacterial gene from *E. coli* that encodes the enzyme β-galactosidase. [95] The precise integration of each donor into the GGTA1 site was demonstrated using Cas9-HDR. Furthermore, LacZ

and human CFTR were persistently expressed in the transduced cells. Moreover, CFTR functionality was demonstrated in cystic fibrosis cells transduced with the HD-Ad vector that delivered the CRISPR-Cas9 system and the human CFTR donor at late cellular passages. [94]

Distinct preclinical studies, both *in vitro* and in animal models, demonstrate the efficacy of CRISPR-Cas9 gene editing in correcting CFTR gene mutations. Nonetheless, no clinical studies have yet been identified to validate these findings. A review of databases that record this type of trial in January 2026 revealed no registered clinical trials employing this tool in patients with cystic fibrosis, [96] indicating that the studies are in preclinical phases. This situation highlights the challenges of applying this gene editing procedure.

## MAIN ETHICAL, TECHNICAL, AND REGULATORY CHALLENGES CONCERNING GENE-EDITING FOR THE TREATMENT OF CYSTIC FIBROSIS

Gene editing, thanks to technologies such as CRISPR-Cas9, is a promising tool in modern medicine for treating hereditary diseases. Though its implementation is not without controversy and significant challenges, comprising the technique's safety, accessibility, ethical implications of modifying the human genome, and the need for adequate regulation to guarantee safe, equitable, and responsible administration. [97, 98] Different issues must be resolved to define an ethical and regulatory framework that ensures its safe and effective clinical application. [99] One of these is the CRISPR-Cas9 efficiency and precision, given the potential for off-target mutations. [98] As a complement, there is a risk of genotoxicity, [99] which could manifest as chromosomal rearrangements (provoked by mutations that alter the number of chromosomes and their composition, the order of genetic material within them, and their interaction with other chromosomes) [100] or chromothripsis (characterized by the shattering of one or several chromosomes into small fragments, followed by random reassembly, resulting in numerous breakpoints and multiple deletions without duplications). [101] Other factors, such as tissue type, cell cycle stage, and individual variability, could affect the specificity of the procedure. [98] In general, these challenges vary depending on the therapeutic purpose, so each pathology requires specific solutions to ensure safety and efficacy.

Beyond the technical challenges, its administration raises ethical considerations. Some are based on the risk of permanently altering DNA that will be transmitted to future generations, especially if the germline is altered. [12]

In addition, there is the hazard of malpractice. Over the past 25 years, the case of Jesse Gelsinger stands out. He suffered from a genetic disorder called ornithine transcarbamylase deficiency and died in 1999 during a clinical trial characterized by ethical violations, highlighting the need for more oversight and rigorous transparency. Similarly, in 2018, it was announced that a Chinese scientist had created genetically edited twins with CRISPR-Cas9 to make them resistant to the human immunodeficiency virus (HIV), generating outrage in the scientific community due to the violation of fundamental principles, as the effects of gene editing on human embryos are still uncertain and risky. [98] Both cases illustrate how economic or scientific interests can take precedence over human rights protection.

The foregoing demonstrates the need for strict international regulation and robust bioethical principles. [102] Legislation is not keeping pace with scientific advances, resulting in outdated regulatory frameworks. Therefore, their constant renewal is urgently desired to ensure the ethical and safe development of these technologies. [98]

## CONCLUSIONS

Cystic fibrosis arises from mutations in the CFTR gene, altering the transport of chloride and bicarbonate across epithelia, leading to thick secretions that obstruct ducts, promote infections, and cause chronic inflammation. As a consequence, there is progressive damage to the lungs and pancreas. Historically, it has been prevalent in the Caucasian population, although reports have been documented in various regions of the world. Traditional treatment focuses on symptom management, so innovative therapeutic options that address the genetic cause have been sought, including gene editing with CRISPR-Cas9.

The CRISPR-Cas9 system has revolutionized genetic engineering by enabling precise modification of specific DNA sequences. In the context of cystic fibrosis, it has demonstrated high efficiency in correcting CFTR gene mutations *in vitro* and in animal models, restoring its function. However, significant challenges related to specificity and the risk of off-target effects exist, hindering its clinical application. Therefore, it is vital to conduct further studies and establish robust regulatory frameworks to ensure safe, effective, and responsible utilization in the future.

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