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# Gene Therapy and Cystic Fibrosis

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**ABSTRACT**

Cystic fibrosis is an inherited disease that mostly has been seen in the Caucasia population [1] but also was found in different ethnicities. Cystic fibrosis affects some organs, especially those that have epithelial cells. Cystic fibrosis disease mainly affects the respiratory and digestive tracts.[2]

Cells that produce mucus, secrete stickier and thicker. This is the main effect of cystic fibrosis disease. While this mucus is stickier and thicker it causes problems in the organs that have a mucus layer surrounding cells.[1] Mainly in the lungs, since the mucus is thicker and stickier, individuals have a problem transporting oxygen to the cells due to the excess amount of mucus on the layer of the lung. That causes patients to have treatment to regulate their blood oxygen levels.

A different story happens in the pancreas. Small tubes that transfer the pancreatic digestive enzymes cannot pass through the tubes due to the blockage on the tubes caused by the thicker mucus.[1] Relatively, digestive enzymes cannot pass through, patients have digestive problems and mostly they face malnourishment.

Since this life-limiting and potentially fatal disease is hereditary, there is no definitive treatment method yet. However, with the developing technology, gene therapy is thought to be the most promising solution for cystic fibrosis patients.

## CYSTIC FIBROSIS

Cystic fibrosis is an autosomal recessive genetic disease that is caused by a gene mutation. This mutation occurs in the cystic fibrosis transmembrane conductance regulator gene, which is also known as CFTR, that is in chromosome number 7.[3] Also, this disease is considered the most lethal genetic disease in the Caucasian population.[4] Since this disease is recessive, both parents must have the disease, or both parents must carry the gene recessively. In that condition, offspring must have both recessive genes together to be born with cystic fibrosis disease.

Depending on the type of mutation, different symptoms may be observed in the patients carrying the CFTR mutated gene. In the same way, the severity of the disease or the severity of symptoms they will show at what age also differs. Patients' diseases are named classic cystic fibrosis when they have two loss-of-function mutations in the CFTR gene. In that case, in patients; it is seen as a chronic bacterial infection in the sinuses, insufficiency of digestive secretes in the pancreas, infertility in men, and excess chloride concentration in sweat. [5]

People with cystic fibrosis have abnormal mucus levels in their lungs and pancreas. This mucus causes congestion, tissue damage, and infection of organs. In addition to the pancreas and lungs, it also causes diseases in organs such as sweat glands, the bile duct, the male reproductive system, and the intestines. [4]

**Pathogenesis**

**Newborn Babies**

Newborn babies that have cystic fibrosis can face a disease called meconium ileus. Meconium is the first stool of a newborn baby.[6] It gets too thick and sticky, so the stool is stuck in the intestines. This situation requires immediate surgical procedures.

**Early Childhood**

During early childhood, cystic fibrosis patients can have pancreatic insufficiency. Due to the thick section, a blockage occurs on the pancreatic duct and pancreatic enzymes cannot be transferred to the small intestine. [7] Therefore, fat and proteins cannot be absorbed sufficiently in the intestine. Over time, it causes poor weight gain and failure to thrive. Eventually, the pancreas gets damaged. The reason for this damage is the enzymes that are stuck in the pancreas due to the blockage start to damage the cells in the pancreas and it causes inflammation. This inflammation is called acute pancreatitis and when it happens over and over, it turns into chronic pancreatitis due to cysts and fibrosis formation in the pancreas. [8] Another important disease that is caused by cystic fibrosis in the pancreas is endocrine dysfunction. It causes insulin-dependent diabetes.[9]

**Later Childhood**

Later in childhood, lung problems start to show up. Patients that have cystic fibrosis start to have lung problems later in childhood.[10] Lung tissue is surrounded by cilia. Those cilia are moving constantly and keeping the mucus away from the lungs. The one other importance of the mucus is keeping some germs such as bacteria. Cilia that are on the lungs are moving the mucus that contains bacteria out of the lungs. This movement is called mucociliary action.[11] However, cystic fibrosis patients have thick mucus so the cilia cannot move the mucus away because of the density. That causes the bacteria that is settled into the mucus to colonize. Colonized bacteria cause infections in patients and they start to show symptoms such as decreased lung function, cough, fever, etc. [10] Antibiotic treatment is used for the treatment of these symptoms but bacteria that are placed into the lungs can be antibiotic-resistant. In that condition, treatment options become limited. Another limiting problem about bacteria that is settled into the mucus is forming a biofilm.[12] This slime-structured biofilm protects bacteria from immune system cells and antibiotics. Therefore, it turns out to be a chronic bacterial infection and inflammation in patients. Airway wall damage causing permanent dilation of bronchi. If the inflammation gets into the blood vessels patients can have hemoptysis which is coughing of blood. Over time repeated cystic fibrosis exacerbations can cause respiratory failure and that can end in death. [13]

**CF in Men**

Cystic fibrosis also causes infertility in men. In men with cystic fibrosis, it has been observed that the testicular tubes, the vas deferens, do not work properly.[14] This situation is explained as the situation where the sperm cannot pass from the testicles to the urethra. In men, this causes infertility. These patients cannot get their partners pregnant naturally, but since there is no problem with sperm development, these couples can have children with different reproductive techniques, such as IVF. [15]

General Pathogenesis of CF

Cystic fibrosis (CF) is an autosomal recessive condition brought on by mutations in the CFTR gene, which codes for a chloride channel protein produced in a variety of tissues. Organs impacted by CFTR failure have abnormal ion balances and decreased mucociliary clearance as a result of poor chloride and water transport across epithelial cell membranes.

Mutations in the CFTR gene cause the respiratory system to produce thicker, dried mucus. Water is less secreted into the liquid at the surface of the airways as a result of the malfunctioning CFTR protein altering the equilibrium of chloride ions. The ensuing sticky mucus blocks the tiny airways and hinders ciliary motion, making it difficult for mucus and inhaled particles to be cleared from the body. Recurrent respiratory infections and the colonization of harmful microorganisms are encouraged by this. In the ensuing persistent inflammation, immune cells are drawn in and activated, causing tissue damage, bronchiectasis, and lung illness that worsens over time.

The exocrine pancreas is impacted by CFTR malfunction in the digestive system, which leads to the generation of unusually thick pancreatic secretions. These secretions clog the pancreatic ducts, preventing the release of the digestive enzymes required for nutritional absorption and digestion. Malnutrition and stunted development can result from the malabsorption of lipids, proteins, and fat-soluble vitamins.

Due to the presence of CFTR protein in many tissues, CFTR malfunction can also impact other organs and systems, including as the liver, reproductive organs, sweat glands, and sinuses. Impaired ion and water transport, abnormal secretions, and susceptibility to infection are the underlying causes.

Depending on the CFTR mutations and other modifying genetic and environmental variables, the severity and clinical symptoms of cystic fibrosis might differ. The goals of treatment plans are to reduce symptoms, control complications, and enhance quality of life. This often entails procedures for clearing the airways, antibiotic treatment, nutritional assistance, and tailored treatments that target certain CFTR mutations.

In cystic fibrosis patients, as shown in Figure 1, [16]sinuses generate inflation and patients live with sinusitis. Due to an excess amount of mucus in the lungs bacterial infections occur plus breathing gets harder. Sweat becomes saltier compared to a healthy person and due to blocked pancreatic ducts, it causes exocrine to unreachable to digestive canals. Besides everything, every patient can have different types of symptoms, and the severity of the symptoms can be diverse.

Oncological risks have been associated with CFTR deficiency. Cystic fibrosis patients have a higher chance of having bowel cancer. It is most likely seen in men than women, but the frequency is not related to age. Cancer can be in the biliary tract, stomach, etc. [17]

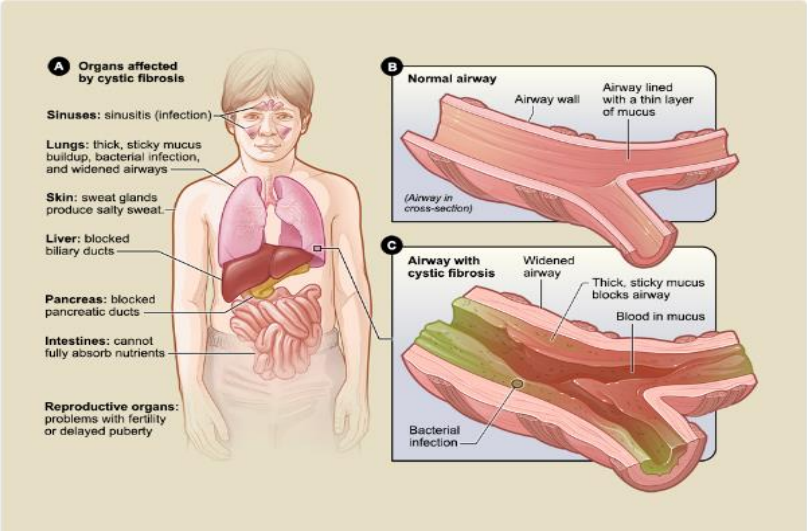



**Figure 1: CF caused by symptoms is shown in the figure. A part shows the general symptoms for patients. Part B shows fully functioning airways for healthy people while part C shows the airways that CF patients have. For other ducts like the pancreatic or liver have the same problem as the airways. ((NIH))**

**Epidemiology of Cystic Fibrosis**

According to the studies, the incidence of cystic fibrosis in people varies depending on race as shown in Figure 2. Also, these studies show that people with cystic fibrosis disease in Asia are the lowest compared to other regions. However, the ratio also differs in Eastern Asia and Western Asia. While the rate of birth of an individual with cystic fibrosis in West Asia is 1/4,000 to 1/10,000, it is 1/100,000 to 1/350,000 in East Asia. [18]

The ratio of birth of an individual with cystic fibrosis is 1/2,560 to 1/15,876 in the middle east. At the same time, researchers state that the number may increase due to consanguineous marriages in this region. [18]

The first reported case in Africa dates to 1959. Although cases were seen in the following years, due to geopolitical situations, poverty, and the increase of viral infections, such as HIV, in Africa, deep enough research could not be done.[18] However, some studies predict that the rate of being born with cystic fibrosis in Africa is between 1/784 and 1/13,924.[19]

The incidence of the disease in North America is 1/2,500 but this rate varies from country to country. While this ratio is 1/2,900 in the United States, it is 1/2,500 in Canada. The reason for this difference is thought to be the fact that there are more different ethnic groups in the United States than in Canada. [18]

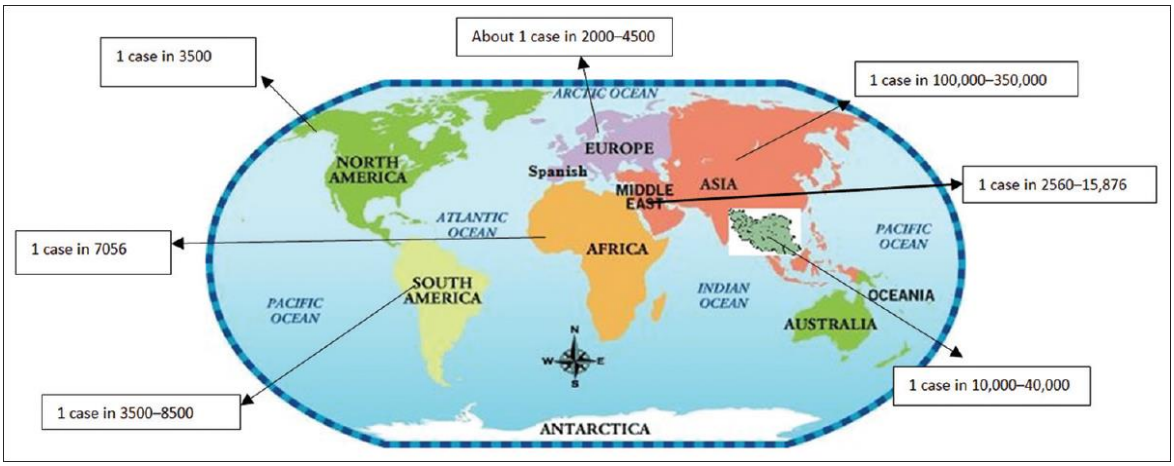



**Figure 2: Figure shows the distribution of Cystic Fibrosis on average depending on country. Asia has the lowest while its highest in Europe. (Geographical distribution of cystic fibrosis; The past 70 years of data analyzis, 2017)**

The incidence of the disease in South America is between 1/3,500 and 1/8,500. Although it differs from country to country, as in other continents, the mortality rate is high due to medical deficiencies and inadequate control of the disease.

Compared to all other continents, Europe has the highest incidence of cystic fibrosis. The highest rate is 1/1,353 in Ireland than in Germany.[18] Finland comes last.

**Diagnostics of Cystic Fibrosis**

In the beginning, the diagnosis of cystic fibrosis was made by pathological investigations. When sweat tests started to be done in general, they are used for diagnosis as well. Chloride levels of cystic fibrosis patients were found to be high in sweat tests.[20] The reason for the high level of chloride in the sweat is that the chloride ions cannot enter the CFTR protein. CFTR protein, which acts as a channel in the cell membrane, takes the chloride ions out of the cell in the lungs and pancreas. But this is different with sweat glands. In normal individuals, chloride ions on the skin are taken through the CFTR channel. However, in

cystic fibrosis patients, because this channel does not work properly, chloride ions cannot be taken into the cell, and therefore their skin becomes salty.

Newborn babies born with cystic fibrosis can have meconium ileus.[6] Which determines the first stool to be stuck in the bowels. Examination of meconium ileus can be done by a bowel examination. The Baby's abdomen becomes rigid and distended. Also, bilious vomiting which means vomiting high bile content can be a signal for meconium ileus.

Tests of close relatives of these people also showed an increase in these levels. In addition to sweat tests, a second test must be done to make a full diagnosis.[21] In that case, lung and pancreatic function tests are performed. Some cystic fibrosis patients may find that the chloride level in their sweat is normal. In that case, a male infertility test is performed because infertility is observed in almost every male individual with cystic fibrosis disease. Evaluation of liver and gallbladder functions, functions of sinuses, and intestinal tests are also sufficient for the diagnosis of this disease. [21]

A nasal potential difference test can be done if the sweat test comes negative. It detects the negative voltage across nasal epithelium associated with cystic fibrosis.[21]

The genotyping method alone is not effective in making the diagnosis. It is known that there are at least 500 CFTR mutations associated with cystic fibrosis. However, genetic tests used in genotyping can only diagnose 70 mutations[22]. Although these 70 mutations can be used to define more than 90% of the cystic fibrosis gene, the absence of some genes is not sufficient to diagnose the disease. In addition to the genotyping, family background can be investigated and sweat tests can be done for the full diagnosis. But it must be known that the most common cystic fibrosis-related mutation can be detected by 2 or more detected genes on both chromosomes.

Exocrine pancreatic function tests also play an important role in diagnosis. When patients respond positively to pancreatic enzyme therapy, doctors can decide that patients have exocrine insufficiency. In this case, tests such as stool enzyme levels, acid absorption, carotene levels, and fat deterioration test are performed. [22] These tests also support the diagnosis of cystic fibrosis. Of course, more than one test is required for a definitive diagnosis.

It is possible to screen for cystic fibrosis in newborns, especially in regions with a high possibility of having the disease. Tests done on newborn babies detect a pancreatic enzyme called immunoreactive trypsinogen (IRT).[17] This enzyme is released into the blood when there is pancreatic damage caused by cystic fibrosis.

As a result, for a clear diagnosis, more than one test is required. Besides genetic testing, supportive testing must be done such as chest X-RAY. Even genetic testing does not necessarily determine cystic fibrosis due to different types of mutations.

**Treatment of Cystic Fibrosis**

For the lungs, clearing out mucus helps with breathing. It can be done with chest physiotherapy and some medications. [23] Medications can be bronchodilators and mucolytics to reduce the density of the mucus surrounding. Mucolytics improve lung functions and decrease inflammation but patients' response may differ. [24]

Anti-inflammatory medicines are also used to reduce inflammation in the lungs.[25] Antibiotics are used to reduce inflammation, but this condition can differ from person to person depending on if the bacteria are antibiotic-resistant or not.

One of the most important pathogens in the lungs is Pseudomonas aeruginosa.[25] In a study, people are

vaccinated for pseudomonas aeruginosa but vaccination failed in the end so scientist does not recommend vaccination of pseudomonas aeruginosa. [24] Instead, inhaled antibacterial is recommended for the infection of Pseudomonas aeruginosa.

Furthermore, bacterial infections are not the only problem for lung diseases. Viral infections and fungal infections also develop lung diseases.

As a supportive treatment, oxygen can be given due to the low blood oxygen level.

Despite all kinds of treatment, when lung diseases increase in severity and turn into chronic diseases, patients may have to have a lung transplant. [17]

Gastrointestinal problems can be covered with some pancreatic enzyme supplements[6] also lipid-soluble vitamins.[6], [26] Patients that have gastrointestinal proteins must have a hypercaloric diet to keep their weight balance and prevent weight loss. Since pancreatic enzymes cannot be transferred to the intestines, patients have trouble digesting nutrients, especially fats. That causes patients to lose weight. To prevent this situation a high-calorie diet is necessary.[17]

In conclusion, the treatment of cystic fibrosis is certainly not efficient since the disease is genetic. Most of the current treatment options depend on symptom treatment rather than permanent treatment. Meanwhile, some gene therapy research is ongoing. It would be a life-changing discovery if gene therapy could be applied to patients for permanent treatment. Also, some therapeutic treatments depend on the mutation type, so they are classified as personal treatments.

## THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR

The CFTR gene encodes a protein called CFTR protein. CFTR gene encoded in DNA transcribed to mRNA during which the introns are spliced out and this happens in the nucleus. The complete process of synthesis of CFTR protein is shown in Figure 3. mRNA is transported to the nucleus and attached to the rough endoplasmic reticulum. Ribosomes take the mRNA molecule and initiate the translation process. This translation happens on the rough endoplasmic reticulum and the protein produced is defined as an immature CFTR protein.

Also, chaperones help protein to fold. After translation, CFTR protein is produced. Therefore, the CFTR protein is transported to the plasma membrane with the Golgi apparatus, and on the membrane, CFTR acts as a channel protein. Most channel proteins are in epithelial cells.

CFTR protein controls the transport of chloride ions. Inside the cell membrane, there are chloride ions ($Cl^-$) and water. On the outside surface of the cell, there is a thick mucus layer. The chloride ions are transported out of the cell by the CFTR protein that is on the membrane and functions as a transportation protein. The calcium ions that are transported out of the cell help the mucus layer that surrounds the cell membrane to become thinner. Also, water molecules inside the cell go out of the cell by diffusion and that also helps the mucus layer to get thinner.

Another function of CFTR protein is to regulate the function of other channels. Such as the transportation of sodium ions.[11]

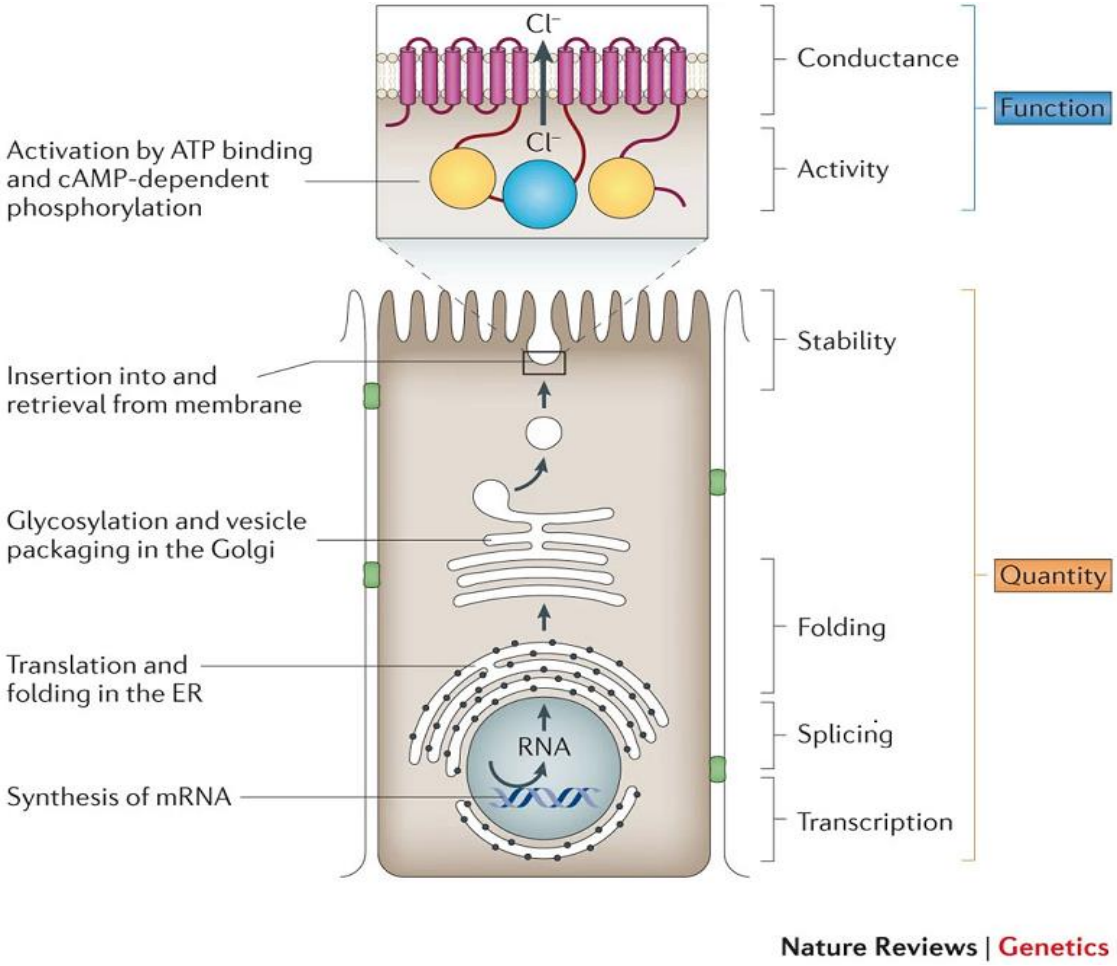



**Figure 3: The synthesis of CFTR protein step by step. Starting from the nucleus, till the protein is placed on the membrane of the cell. (Cystic fibrosis genetics: from molecular understanding to clinical application, 2014)**

**Structure of Cystic Fibrosis Transmembrane Conductance Regulator**

The CFTR protein is a complex glycoprotein composed of 1480 amino acids [3] and consists of 5 domains (see Figure 4). Two of them are membrane-spanning domains, in short MSDs. Two of them are nucleotide-binding domains, in short NBDs. Lastly, a regulatory domain contains phosphorylation sites, in short R. The movement or conductance of $Cl^-$ across the CFTR protein requires phosphorylation of the regulatory domain. Also, binding and hydrolysis of ATP are required for the opening and closing of the CFTR channel. Membrane-spanning domains provide the formation of pores in the canal. When the R domain is phosphorylated, it determines the channel activity. Nucleotide-binding domains control the transitions along with ATP hydrolysis. [27]

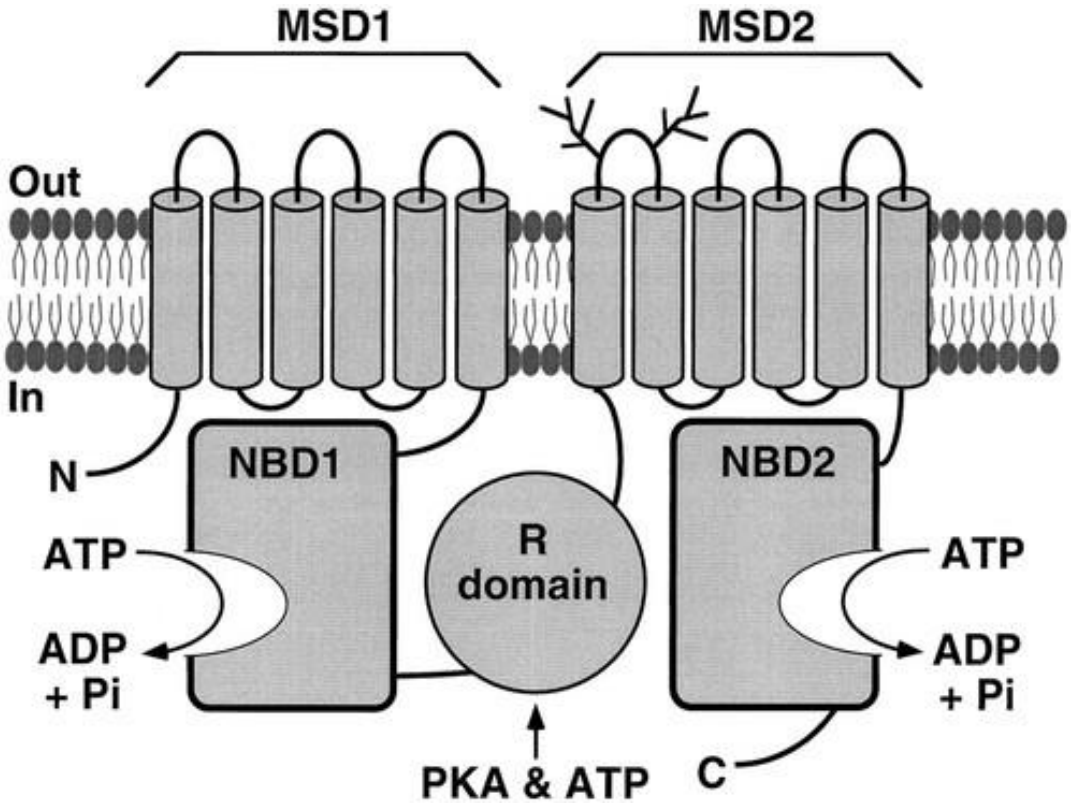
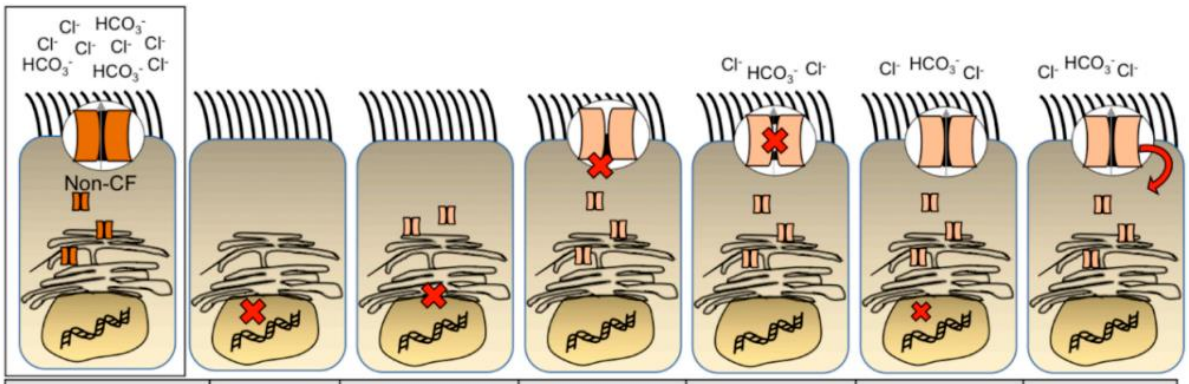



**Figure 4: NBD2 define nucleotide-binding domain1 and nucleotide-binding domain 2. MSD1 and MSD2 define membrane-spanning domain 1 and membrane-spanning domain 2. Figure shows the domain structure of protein CFTR. The R domain defines the regulatory domain. NBD1 and (Structure and function of the CFTR chloride channel, 1999)**

**Mutations**

The most common mutation that occurs in the CFTR gene is called ΔF508. [28] Δ refers to deletion, F refers to Phenylalanine, and 508 refers to the 508$^{th}$ amino acid out of 1480. When there is a mutation in the CFTR gene, the CFTR gene can still be transcribed to mRNA and can be attached to the rough endoplasmic reticulum and ribosomes can translate the gene into protein. But the dysfunction during this process is, produced protein cannot be transported to the cell membrane due to the misfolding of the protein. This misfolding is called impaired post-translational processing. So that the Calcium ions cannot be transported to the outside of the cell membrane and the mucus stays thick and sticky.

6 types of different mutations exist for CF patients. [29]All these mutations cause different types of deficiency in the protein (see Figure 5). Mutation type one causes no synthesis of the CFTR protein while 6$^{th}$ one is partially functioning. Some medical therapies are applied depending on this mutation type. Such as, when there is no synthesis on the gene itself, then a functioning gene can be transferred into a gene for a fully functioning CFTR gene. When there is a misfolding of the protein, then some medications to improve folding can be supportive for the patients.




| Class of CFTR mutation | I | II | III | IV | V | VI |
|---|---|---|---|---|---|---|
| Protein output | No synthesis | Block in processing | Block in regulation | Reduced conductance | Reduced synthesis | Reduced half-life |
| Mutation example | G542X | F508 | G551D | R117H | 3489+10kb C>T | 4326delTC |
| % of people with CF who have at least one mutation* | 22% | 88% | 6% | 6% | 5% | 5% |
| Small molecules** | | VX-809,VX-661 correctors | VX-770 potentiator | | | |

**Figure 5: CFTR mutation classes are divided into 6 based on the function resulting from the mutation or protein output. Regions indicated in red indicate the effect points of the mutation. Percentages were determined according to the data in the United States (2017) and more than one mutation can be seen in a single individual. (Cystic fibrosis gene therapy: Looking back, looking forward, 2018)**

All these different classes might show different severity or symptoms in cystic fibrosis patients. It even determines at what age which symptoms will show. Some patients do not show any symptoms till adolescence meanwhile some start when newborns. This classification helps for further research and possible future treatments for the patients.

## GENE THERAPY

Gene therapy is simply the process of gene silencing, gene addition, gene editing, or replacement of the genetic material in a cell. This change causes the gene in the cell to be repaired or the gene that causes the disease to be silenced or removed. As a result, the product of the harmful gene is reduced or the gene is replaced by a functional gene. [30] Often, especially in cancer treatments, most of the experiments are done by adding genes.[31] Gene therapy also plays a major role in the development of vaccines for infectious diseases and cancer by using viral vectors. [31]

Gene therapy is promising not only for cancer treatments but also for all other hereditary diseases. Over 10 years, (data shown in Figure 6 between years 2010-2020) most of the clinical trials done are related to the cancer even though gene therapy is a treatment for genetic diseases.[32]

Gene therapy is mostly using the delivery of vectors. Nanostructures, plasmids, and the viral genome are the most commonly used ones. [33] Especially, viruses compared to others are more used for therapeutic purposes due to their ability to attach to host cells and manipulate by transferring their genetic material and can cause activation or inhibition of the host cells. Also, the viral genome has a small genome size and is simple to study.[34] Therefore, most of the clinical research is done by using viral vectors.

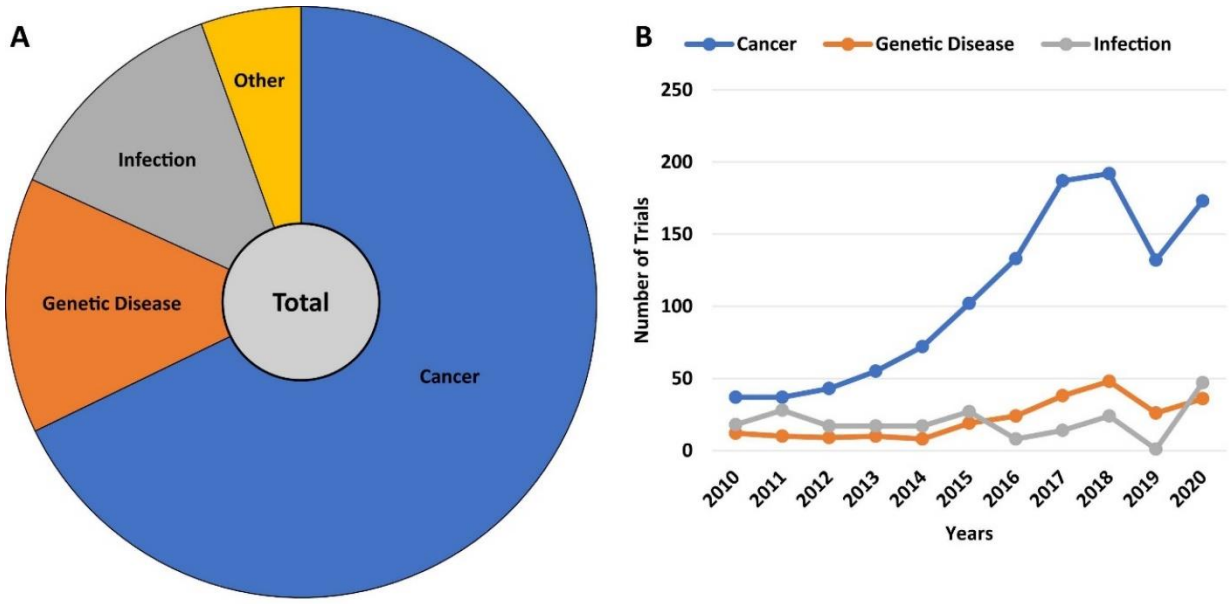



**Figure 6: The rate of clinical trials done regarding of different type of diseases. This data shows the clinical trials done between the year 2010-2020. (Gene therapy clinical trials, where do we go? An overview, 2022)**

## Methodologies

There are two main methodologies in gene therapy applications: *in vivo* and *ex vivo* (figure 7). [35]

*In vivo*, treatments are procedures where the vectors are directly transferred to the patient's body. *Ex vivo* treatments are the procedures where the specific cells of the patient (or a donor) are cultured in a laboratory and addition of vectors on the collected sample. Then cultured cells are replaced in the patient's body.

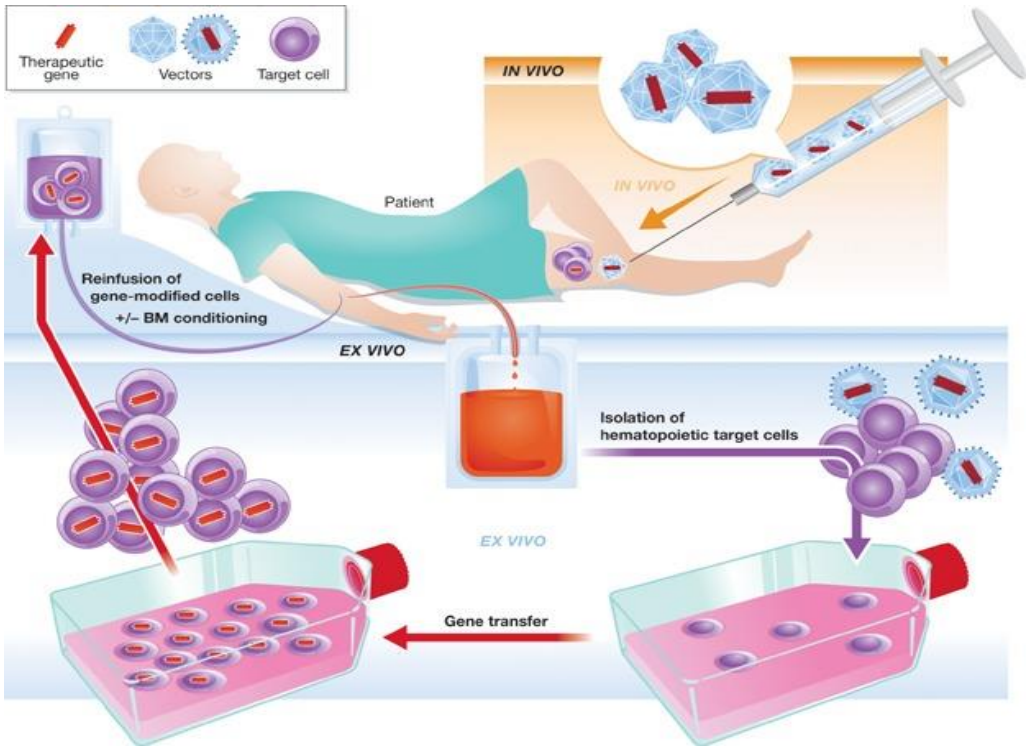



**Figure 7: schematic view of in vivo and ex vivo applications. In vivo, direct transmission of the therapeutic gene to the body. Ex vivo, first cell was isolated, modified in the cell culture, and transferred to the patient. (Gene therapy on the move, 2013)**

*In-vivo* method is used when cells cannot be separated from the body, so they are more suitable for use in tissues that are easy to contact. It also does not require much laboratory work. However, the inability to make direct contact with the desired cells and the possible response of the immune system are the disadvantages of the in vivo method.[32]

On the other hand, the *ex-vivo* method allows the vector to come into direct contact with specifically cultured cells and to create the most suitable environment for the culture.[32] But it requires much more equipment and well-equipped laboratories.

Vectors

Mainly divided into viral vectors and non-viral vectors.

Viral Vectors

Viruses are made up of nucleic acids, envelope structure, and capsid protein. While applying gene therapy, mostly infectious agents are blocked to prevent the infection. Also, that prevents cytotoxicity and increases the therapeutic efficacy. [36] DNA and RNA vectors cannot enter the cell nucleus without any trigger. However, viral vectors can enter the cell with their tiny structure and the mechanism they have developed as a result of evolution. [34] This enables viral vectors to be used as the most promising for gene therapy. Virus-like particles, retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses are mainly used for applications.[37] Adenovirus vectors and adeno-associated vectors (AAV) are the most common viral vectors.

Adenovirus vectors are the most studied and published vectors due to their gene expression level and the ability to big diversity of host cells.[35], [36] Adenovirus vectors are used for the treatment of cancer and vaccines.[38] Adenoviruses do not insert into the host cell genome and are therefore considered safe, but are not preferred recently due to the possibility of causing inflammation and some cases virus being inactive.[34]

Adeno-associated virus vectors can safely cure the RPE65-defected gene which causes retinal degradation and blindness. After clinical trials, patients have better eyesight. Also have been used for the deficiency of the blood-clotting cascade. [31]

Non-Viral Vectors

Non-viral vectors can be lipid-based nanoparticles, polymers, lipid-polymer hybrids, and inorganic materials.[39] Mostly used for vaccine production. Such as mRNA-based lipid nanoparticles and these vaccines can have good efficiency. Viral vectors are mostly used in research. Although, non-viral vectors produce less cytotoxicity and cause less mutagenesis. However, non-viral vectors are not effective as viral vectors and also the safety issues. [40]

## GENE EDITING

Gene editing, especially with recently developed biotechnology, has great potential for improving cures for genetic diseases and understanding human genetics. In the last century, with the discovery of sequencing the human genome, technologies for determining and analyzing expressions in genes have advanced. In this way, genetic diseases can be diagnosed more easily, and genome sequences can be investigated more comprehensively.

Gene editing makes it possible to modify the genetic material of organisms by using developed biotechnological techniques. The primary purpose of this regulation in medicine is to cure genetic mutations or diseases but also can be used to give desired characteristics. For other organisms, it can be used to develop for it to be beneficial to humans by changing the genetic materials.[41]

In medicine, many genetic diseases currently do not have a cure, but gene editing has great potential in correcting these mutated genes and is thought to provide a permanent treatment.[42] Although genetic diseases in humans are more complex in the genome, some of the common genetic diseases are caused by mutations in a single gene (monogenic).[43] Cystic fibrosis is one of these monogenic diseases.[44] The known number of monogenic diseases is approximately 5000 and 250 million people are affected by monogenic diseases.[45] So on, monogenic diseases can have different genotypes meaning that diseases' severity can differ from patient to patient.

Another usage of gene therapy can be in agriculture. It can be used to grow plants that are more resistant to environmental factors.[46] In addition, it can be used to increase diversity by using endangered animals. [47] It can also be used to reintroduce extinct species and to facilitate their adaptation to a new environment.[48]

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)

CRISPR stands for clustered Regularly Interspaced Short Palindromic Repeats and it's one of the most important technology used in gene editing technology. CRISPR enables us to cut genetic material precisely in living cells. Normally, bacteria and archaea use this process naturally as an adaptive immune system mechanism; the purpose of this mechanism is to protect themselves against viruses and plasmids..[49]

Bacteria become resistant to the next infection by inserting the genetic material from previous viral infections into their DNA. The bacteria infected by the virus or plasmid insert a small size of the viral genome or plasmid into the bacterial genome using the Cas protein.[50] In other words, the Cas protein acts as a molecular scissor. The viral genome is then transcribed into RNA.[51] This RNA molecule then guides the Cas protein from which part of the viral genome must be cut on the later infections.

Cas (CRISPR-associated system) proteins and RNA are used in this system. Cas protein is used to cut and modify DNA. The Cas protein is encoded near a location called the CRISPR locus.[52] The RNA molecule is called guide RNA (gRNA).

This system is used in biotechnology to cut the desired part of genetic material by using the Cas protein. This is done by designing a gRNA molecule according to the targeted gene sequence, allowing precise modifications to the genome.

The gRNA is designed to be complementary to the target sequence. As shown in Figure 8, this gRNA located inside the Cas enzyme, directs the enzyme to the complementary position on the DNA. Two catalytic domains on the Cas enzyme cleave at the targeted site, creating a double-stranded break. [53]

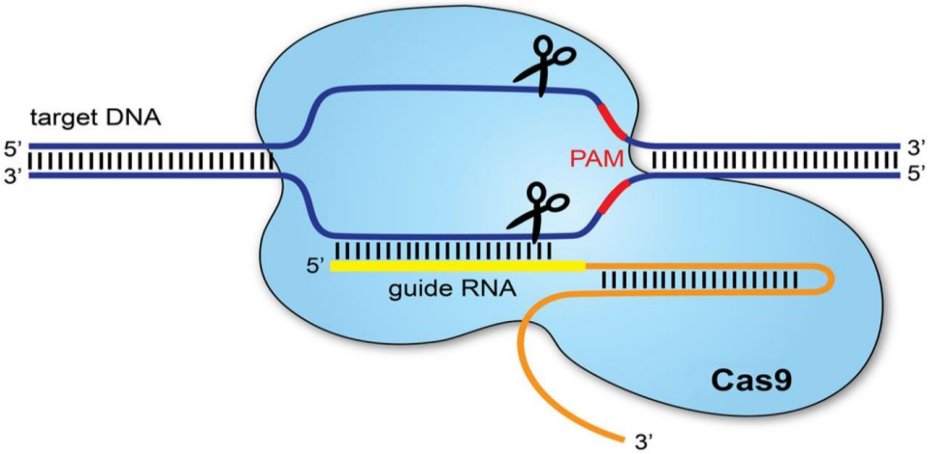



**Figure 8: Cas9 enzyme attached to DNA by the guide RNA. PAM (protospacer adjacent motifs) is a nucleotide sequence close to the complementary sequence to RNA and is the starting sequence for binding RNA to DNA. (What is CRISPR/Cas9?, 2016)**

The repair process is triggered due to the double-strand cut by two catalytic domains and can be used for genome editing.[50] There are two primary types of repair mechanisms (see Figure 9): non-homologous end joining (NHEJ) and homology-directed repair (HDR).[54]

NHEJ is the joining of cut ends of DNA. Results in insertions and deletions in the cut region.[55] Thus, the gene in that region becomes unreadable or may be a result of the synthesis of a dysfunctional protein.[56] However, it can result in unwanted mutations in the DNA.[56]

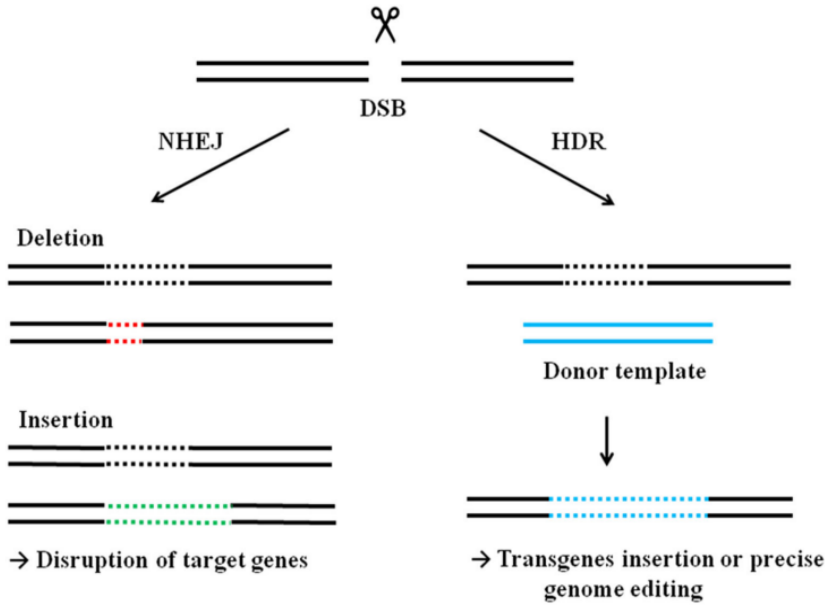



**Figure 9: Engineered nuclease-induced genome editing. DNA repair takes place following the formation of a double-stranded break (DSB) through non-homologous end joining (NHEJ) which results in some deletions or insertions by disruption of target genes, or homology-directed repair (HDR) which includes a donor DNA template for precise genome editing or transgenes insertion. (CRISPR/Cas9 Immune System as a Tool for Genome Engineering, 2017)**

HDR is less common than NHEJ, and this repair mechanism uses a cell-introducible repair template.[55] Therefore, it is often used to correct point mutations or insert a designed DNA sequence.[57] This method is more precise than NHEJ but less efficient.[56]

CRISPR Systems

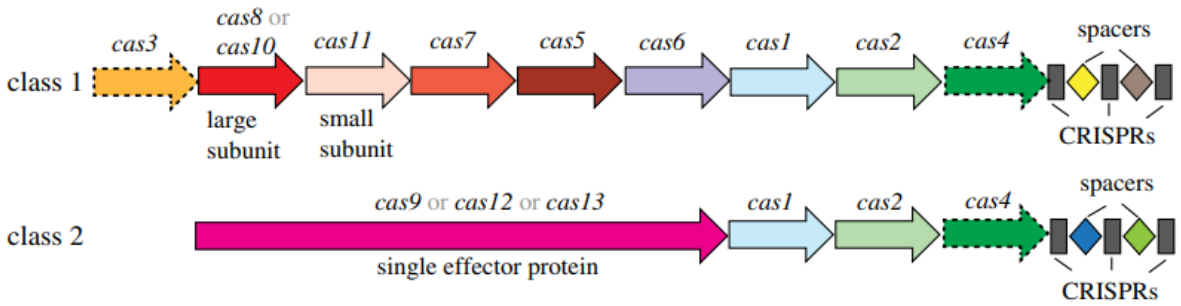



**Figure 10 Class 1 and Class 2 CRISPR-Cas systems and their features, and organizations. Genes indicated as arrows. (Origins and evolution of CRISPR-Cas systems, 2019)**

CRISPR systems are divided into two groups according to their structure and mechanism: Class 1 and Class 2 (Figure 10). There are six types of CRISPR/Cas systems and over thirty subtypes.[58] Class 1

systems include types I, III, and IV and are more complex due to their multi-subunit nature, using multiple proteins to target and cleave the DNA. Class 2 systems include types II, V, and VI and use a single protein. It is simpler in structure and uses a single protein to target and cleave the DNA.[59], [60] Class 2 systems are more widely used in biotechnology due to manipulation of the gene is easier, yet class one systems are more abundant in nature.[60]

*Class 1 CRISPR Systems*

Type I is the most complex and largest subtype in the CRISPR systems. It contains crRNA (CRISPR RNA) molecules with multiple Cas proteins and this complex effector Cas proteins called Cascade (CRISPR-associated complex for antiviral defense).[61]

Type III is a complex multi-subunit effector called Csm (CRISPR-associated complex for the interference of target RNA) for DNA manipulation. This complex can be Cas10 or Cmr4.[62]

Type IV complexes contain a crRNA assembled with Cas7, Cas6, and Csf1.[60]

*Class 2 CRISPR Systems*

Type II is the most widely used and known system in CRISPR technology. It uses the Cas9 effector protein. crRNA identifies the relevant site on the DNA and Cas9 cuts the region.[58]

Type V uses the Cas12 effector protein along with the crRNA, but Cas12 produces a staggered cut site on the DNA.[58], [63]

Type VI uses Cas13 effector protein along with the crRNA, but unlike other types, the target sequence is RNA, not DNA.[64]

Transfer Methods

Transfer of genetic material is referred to as transfection. Transfection during CRISPR applications can be done in three formats: DNA, mRNA, and RNP (ribonucleoprotein).[65] RNP is a complex of RNA and RNA-binding proteins. CRISPR requires delivery of the CRISPR/Cas9 complex into the cytoplasm and that requires this complex to enter the cell membrane first and then the nuclear membrane.

Before editing the genome, DNA-format vectors require transcription and translation. It can be introduced into cells through transfection or electroporation. DNA entered the cytoplasm and is first transcribed into gRNA and mRNA that will later be used to code the Cas9 protein.[66]

mRNA format is directly translated into the cytoplasm and then enters the nucleus. This mRNA molecule carries instructions to cells to produce Cas9 protein. That means it does not require a transcription process.[67]

RNP, ribonucleoprotein, is the meaning of its name, it is a complex of Cas9 and gRNA so it does not require transcription and translation process.[66] This complex can be introduced into cells through electroporation and microinjection. That makes this format to be more efficient compared to the mRNA format and DNA format.

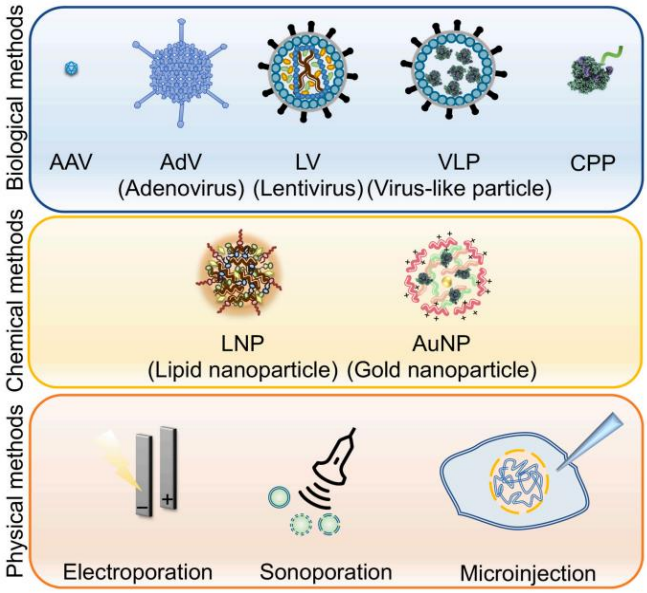



**Figure 11: Delivery tools divided into three and methods are shown. AAV indicates Adeno-Associated Virus (Delivery of CRISPR-Cas tools for in vivo genome editing therapy: Trends and challenges, 2022)**

Biological Transfection

As mentioned in the viral vectors part, adenoviruses, AAV, retroviruses, and lentiviruses are used for biological transfection. Viral transduction is the transfer of a viral vector in the host cell. That application has a limitation due to packaging size due to the use of viruses and each virus has a different capacity.[68] Also, some toxicity caused by the viral genome can be a problem due to immune response.[69]

Chemical Transfection

Chemical transfection mostly uses lipid-based and polymer-based transfection reagents. [70] These vectors include liposomes and lipid nanoparticles or synthetic polymers. Chemical vectors' delivery mechanisms are based on the encapsulation of genetic material, fuses with the cell membrane through endocytosis, and release as cargo inside the cell.[71] Therefore, genome size is not important.

Physical Transfection

There are several physical transfection applications. These are electroporation, microinjection, and sonoporation.

Electroporation is the application of electrical pulses to the cells and electrical signals create temporary pores on the membrane. Those pores create access to the cytoplasm for RNP and plasmid DNA. [65]

Microinjection is the direct injection of genetic materials inside cells or nuclei by using a glass micropipette. [72]There is no cargo limitation for microinjection processes, and it is done under a microscope.

Sonoporation is similar to electroporation but instead of the usage of electrical pulses, it uses ultrasound waves to create pores on the membrane. Genetic materials are injected with microbubbles or microdroplets with ultrasound waves. [73]

Advantages and Limitations

Transfections are promising but each of them has some advantages and limitations. Cargo size and immune system reactions can be a limitation for biological transfections.[74] Even though there are

different types of viral vectors, for some applications there is limited specificity because of the diversity of cell types. Also, for using viral vectors, manufacturers need to produce and purify the viral vectors. This process is time-consuming and requires large-scale production.[75] That causes an increase in the cost of viral vectors.

Biological transfections also offer several advantages. The ability of viral vectors to enter the cell and insert the vector into the host cell is their natural ability, so that, this increases the transduction efficiency.[75] The fact that this process is natural, also ensures that the application is more stable and long-term.[74] Another advantage is that viruses select the host cells according to their receptors. By designing promoters of viruses, the number of the diversity of host cells can be increased.[75] At the same time, engineerability can be applied depending on the need to increase efficiency. In addition to these, the most important feature of viral transfection is its ease in in-vivo applications.[65]

Chemical transfections require some reagents for applications. These reagents can be toxic for cells especially when applications are done with high concentrations and can cause some unwanted reactions with other components inside the cell. Another problem is, every cell has a different reaction for different applications, and that causes a difference in efficiency and toxicity.[76] One of the chemical transfection methods is lipofection and this process has a limitation due to plasmid DNA can enter the cytoplasm but cargo size does not allow the DNA to reach into the nucleus.[65]

Chemical transfections are generally easier to implement. Chemical transfection is the addition of reagents and gene-editing tools to target cells.[70] In this way, a successful application can be achieved even if the researchers have different levels of expertise. Although cytotoxicity can be a limitation, the immune system response is less compared to viral vectors.[76] Adjusting the amount of incubation time of the reactors also has the potential to reduce cytotoxicity. Another advantage is the low cost.[77] In particular, it is less costly than physical and biological applications, so it is more accessible to make applications at a larger scale.

Physical transfection can affect the sensitivity of some cell types because some cells are sensitive to physical applications and that can affect the successful transfection. This process also requires some special equipment. Optimization of the environment of the cells and well-qualified expertise can be a requirement. For example, microinjection requires a very sensitive application and since it’s very small sized, it’s hard to apply this process for a high number of cells.[72] Physical applications require physical force, therefore parameters like pulse strength, time, and pressure must be calculated carefully.[78] Physical transfection also requires physical contact for gene delivery so that in vitro applications can be done easier but in vivo delivery technique is harder to apply since it’s harder to do direct contact.

One of the most prominent advantages of physical transfection is the rapid and efficient intracellular delivery of genetic material.[73] The reason for this is that this method is applied by direct contact. At the same time, applicability to cells is thus versatile. Physical treatments do not contain viral particles and thus less immune reactions.[79] Also, in physical applications, the parameters of the physical forces can be adjusted and the efficiency can be increased.

## GENE THERAPY AND CYSTIC FIBROSIS

Cystic fibrosis is a life-limiting and quite common genetic disorder caused by a CFTR gene mutation. Dysfunction of the CFTR gene causes an abnormality of chloride channels. Although the severity of this disease varies from person to person, it causes symptoms such as lung diseases, malnutrition, sinusitis, and liver and pancreatic dysfunction[1]. There is no cure for this disease, so all therapy applications are

done to treat or make symptoms milder. However, this disease is a disorder that limits the life of patients and reduces their quality of life.

With the developments in biotechnology, gene therapy applications have been the most promising treatment option for the treatment of CF. Gene therapy offers the potential to correct mutations in the CFTR gene and a normally functioning CFTR gene can be obtained, providing the potential to get a properly functioning CFTR protein. There are two main applications in gene therapy.[80] One of these applications is gene replacement. Gene replacement applications are made by delivering a properly functioning CFTR gene into the cell. Gene delivery is generally transferred by viral vectors such as AVV or lentiviruses.[29] After viral vectors transfer, the normally functioning CFTR gene into the cell, protein synthesis is expected in the cell and a properly functioning CFTR protein is expected to be obtained. As a result of this application, it is expected that the symptoms in the patients will be milder.[81]

The other application is gene editing. Gene editing aims to directly edit the faulty gene sequence of CF patients. After DNA analysis of patients, the mutated gene is determined. Once the defective sequences of the CFTR gene are identified, the gene is rearranged precisely. It is expected that this application will result in more precise changes being made than gene replacement.[82] Although these applications are promising for patients, they also bring a lot of problems. Pre-and post-clinical studies have been conducted, and some cases have shown improvements in lung function and a reduction in symptoms [83]. In this way, the rate of hospitalized patients has decreased.

The success rates of the applications and the improvement potential are directly proportional. Gene transfer and gene editing applications must be successful and changes within the cell must be applicable. For this, optimization of viral vectors is important. In treatments involving viral vectors, the immune system must always be considered, because the immune system may recognize viral vectors as a threat and reduce the cure rate and even cause further complications.[84]

At present, the potential gene therapy of cystic fibrosis is one of the most emphasized topics of researchers, and maximizing the therapeutic effects of gene therapy, obtaining stable and long-lasting gene expression, and maximizing immune reactions[80] are the most important goals.

## CLINICAL TRIALS, SAFETY, AND FUTURE

Clinical trials in general are of paramount importance for treatments and are important for recognizing the disease and developing new treatments. These trials are needed to explore potential treatments and test their safety and efficiency. The effectiveness of most available treatments in patients is different and the primary goal is to improve these results, find the safest treatment, and improve the quality of life of patients. At the same time, the data published after trials are a guide for further research.

Clinical Trials of Cystic Fibrosis

In clinical trials of CF patients, the evaluation of the tolerability, safety, and efficiency of drugs designed according to the complications experienced by the patients is investigated. These assessments are often done for people with cystic fibrosis to improve lung function, monitor nutritional status, and investigate effects on quality of life.

There are several different approaches in clinical trials of CF. These are drug therapies, gene-based therapies, and supportive therapies. Drug-based therapies include small molecule drugs, function modulators of the CFTR proteins, or anti-inflammatory agents. Gene-based therapies are gene therapy, gene editing, and RNA-based therapies.[85] Gene-based therapies aim to regulate the function inside the cell. Supportive therapies are more symptom-based and are the treatments most used in hospitals at present.

Such as physiotherapy to regulate lung function or nutritional support or antibiotic treatments to reduce inflammation.

Problems encountered in clinical trials usually arise from the fact that the mutations that cause disease in CF patients are different from each other because the results of different mutations of this disease are also different. It also causes statistical problems that require finding participants and long-term follow-up. Additionally, appropriate control groups, ethical issues, and adequate funding for trials are challenges.

Prominent CF Clinical Trials

Another promising treatment for CF is CFTR modulators. CFTR modulators are drugs aimed at improving the function of the dysfunctional CFTR protein. G551D, a CFTR mutation, can be treated with a modulator called ivacaftor. Clinical trials on Ivacaftor have shown significant improvements in lung function and improvement in weight gain.[86]

Lumacaftor-Ivacaftor are synergistic CFTR modulators used to improve the function of CFTR protein that is dysfunctional due to the most common mutation F508del. [87] This combination shows improvement in patients' lung functions.

The Tezacaftor-Ivacaftor combination, like Lumacaftor-Ivacaftor, is the modulator for correcting the function caused by the F508del mutation. This combination shows improvement in patients' lung functions better compared to Ivacaftor only.[86]

Gene-based therapies aim to directly or indirectly treat the mutated CFTR gene. Early-phase clinical trials of gene therapy are showing promising results such as improvement in CFTR function.[88] There are two types of gene therapy. In non-integrated gene therapy, a fully functioning CFTR gene is transferred to the patient. Since the transferred gene is not integrated into DNA, it is not permanent, and patients need to take the treatment repeatedly. Integrated gene therapy is the delivery of a DNA fragment with the CFTR gene into the cell and is permanent. However, this can also result in the integration of the DNA fragment into unwanted regions of the genome and cause various complications.

On the other hand, currently, there are no gene editing applications for the treatment of CF.[89] One of the reasons is that gene editing requires the exact location of the mutation point to be determined, but many mutations cause CF. It should also be considered that there may be errors during gene editing applications. A wrong change in DNA can cause undesirable consequences such as cancer. There are currently a lot of studies working on gene editing, but this research is currently being done on cells and animals.[89] For gene editing applications to start in humans, the application must be safer.

Safety and Regulatory of CF Clinical Trials

In general, clinical trials are conducted under strict supervision to ensure patient safety and ethical issues. Institutions such as the US Food and Drug Administration (FDA), and the European Medicine Agency (EMA) create guidelines for trials.[90] Its general objectives are important for situations such as the protection of human rights and the fair distribution of research. In particular, the safety of patients is very important for clinical research. There are strict guidelines for identifying and mitigating potential risks, and patients are informed of any possible situation. Patients taking part in clinical trials must have accepted the potential benefits and risks. At the same time, patients can withdraw from clinical trials if they wish.

Clinical studies are essential to improving our knowledge of and ability to treat cystic fibrosis. They offer a setting for evaluating cutting-edge treatments, enhancing patient outcomes, and directing the direction of CF care in the future. Clinical trials aid in the creation of specialized, targeted medicines for CF patients through careful consideration of safety, stringent monitoring, and moral behavior. The continued progress and promising prospects in CF research give people with this difficult genetic illness hope for an improved

quality of life and outcomes.

## PURPOSE

Cystic fibrosis is an inherited and life-limiting disease that is mostly seen in European descendants but has been observed at different rates worldwide. As with most genetic diseases, there is no definite treatment, and it can even be fatal in some patients. Although the approximate lifetime for cystic fibrosis patients increased over time, still there are a lot of cystic fibrosis patients who suffer from this.

In some patients, symptoms that begin in infancy may begin at older ages. If the disease shows symptoms at a later age, these patients show that they experience cystic fibrosis less intensely than other patients. This depends on the type of mutation in the CFTR gene. In some mutations, the CFTR protein can function partially, resulting in patients with milder symptoms. Studies on gene therapy, methods that will alleviate the symptoms of cystic fibrosis patients, even those with severe disease, or that can permanently treat the disease with gene editing.

## CONCLUSION

After the human genome project, the focus of researchers has changed for 20-30 years to search and detect the disease-causing genes in this genome map and to use these diseased genes for treatment by changing them with various methods. Permanent treatment or reduction of the effects of genetic or infectious, hereditary, or mutational diseases is especially important for a society with an increasing life expectancy. Today, there are still too many people whose quality of life and duration of life are reduced due to genetic diseases. At the same time, research is carried out to treat infectious and incurable diseases such as AIDS. This not-so-old gene therapy gives hope that people with these diseases can have a normal life. It is being investigated that, genetic diseases, such as cystic fibrosis, which causes high mortality, especially in individuals of European origins, can also be treated with gene therapy. Although clinical trials have started in the last 20-30 years, they still cannot be used reliably, but researchers state that gene therapy will be used in the treatment of some diseases in 2-25 years, with a reliable and high outcome rate.[31] The bottom line is that once you identify which gene causes a disease, it can be possible to be treated with genetic engineering.

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