The Use of CRISPR-Cas9 Genetic Modification as a Treatment for Huntington's Disease

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Colegio Granadino

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Department of Humanities

Senior Independent Project

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Isabela Osorio Arbeláez

Resumen

La enfermedad de Huntington (EH) es un trastorno neurodegenerativo poco común en la población que ha preocupado al sector de la salud y ha afectado negativamente la calidad de vida de sus pacientes. Se hereda de forma autosómica dominante y se debe a una repetición extensa del trinucleótido CAG en la localización citogenética 4p16.3 del gen huntingtina (HTT). Si bien existen tratamientos que alivian sus síntomas, como medicamentos y apoyo psicológico, aún no existe una cura definitiva. CRISPR-Cas9 es una herramienta de edición genómica comúnmente utilizada en la investigación biomédica que promete tratar o curar la EH, pero su uso como tratamiento aún se encuentra en fase de investigación. Este proyecto de investigación analizó la eficacia de implementar CRISPR-Cas9 como tratamiento para la EH a mayor escala.

El trabajo empleó un enfoque teórico basado en artículos científicos e investigaciones previas para analizar los procesos genéticos y moleculares que subyacen al desarrollo de la EH, el procedimiento paso a paso y las aplicaciones de CRISPR-Cas9, y el éxito de experimentos previos *in vivo* e *in vitro* con CRISPR-Cas9 en modelos de la EH. En segundo lugar, la tesis empleó un método pragmático basado en entrevistas semiestructuradas con especialistas en neurología, genética y biotecnología. Esta investigación tuvo como objetivo aportar nuevas perspectivas y ampliar el conocimiento previo sobre el tema, con el fin de impulsar el uso futuro de la tecnología CRISPR-Cas9 en pacientes con la EH como tratamiento o cura de la enfermedad.

Palabras clave: enfermedad de Huntington, CRISPR-Cas9, modificación genética, neurodegeneración, tratamiento.

Abstract

Huntington's Disease (HD) is a rare neurodegenerative disorder that has concerned the medical field and negatively impacted the quality of life of its patients. It is an autosomal dominantly inherited disease that is caused by an extensive repeat of the trinucleotide CAG on the cytogenetic location 4p16.3 in the Huntingtin (HTT) gene. Even though there are treatments that ameliorate its symptoms, such as medications and psychological support, there is still no definitive cure for the disorder. CRISPR-Cas9, a commonly used genome-editing tool in biomedical research, holds promise to treat or cure HD, but its use as a treatment is still in the process of investigation. This research project analyzed the efficiency of implementing CRISPR-Cas9 as a treatment for HD disease on a larger scale in medical centers.

The project used a theoretical approach based on scientific papers and previous research to analyze the genetic and molecular processes underlying the development of HD, the step-by-step process and applications of CRISPR-Cas9, and the success of past *in vivo* and *in vitro* experimentations of CRISPR-Cas9 in HD models. Secondly, the paper used a pragmatic method focusing on semi structured interviews with specialists in neurology, genetics, and biotechnology. This study aimed to provide insights and contribute to the previous knowledge on the topic, to accomplish the future use of CRISPR-Cas9 technology for HD patients as a treatment or cure.

Key words: Huntington's Disease, CRISPR-Cas9, gene editing, neurodegeneration, treatment.

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Justification

Modern biotechnology appeared in the late nineteenth century and started involving genetic manipulation after Francis Crick and James Watson discovered the double-helical structure of DNA. Since then, scientists began to unravel the genetic code, discovering the inherent molecular machinery, DNA technologies, and genetic engineering methodologies. These tools hold promise for significant advancements in the medical field because they enable gene modification, new disease treatments, more profound research of DNA, and drug and vaccine development. It is crucial to raise awareness about these new mechanisms, as they provide possible cures to medical illnesses, contributing to improving worldwide quality of life. Moreover, as critical-thinking individuals, it's pivotal to investigate the precision and effectiveness of these tools because they will continue to be incorporated into the public health system.

In clinical pathology, the most concerning illnesses have frequently included neurological disorders and specific neurodegenerative diseases, such as Alzheimer's Disease, Parkinson's Disease, and Huntington's Disease. The adequate functioning of the brain is essential for survival because it is a central control system for body functions like breathing, heart rate, blood pressure, digestion, and locomotion. The nervous system also regulates emotions, stores information, and allows conscious thinking, sensory processing, and communication. The progressive atrophy of neurons and tissue in neurodegenerative diseases leads to failure in these vital functions, turning this into an issue that must be addressed immediately. In addition, the increasing prevalence of these conditions and their profound impact on the individuals' and their families' quality of life has turned research for new therapies into a necessity. Furthermore, the brain remains the least understood organ in the human body, a fact that has long intrigued

researchers and was a catalyst for further exploration and inquiry in this study. Recent biotechnological advancements, particularly CRISPR-Cas9, can be useful in treating neurodegenerative diseases that are genetically inherited, such as Huntington's Disease, which is why it's crucial to analyze this treatment option.

This research topic intersected two fields that hold significant relevance for the advancement of professional disciplines: medicine and technology. The medical discipline not only plays a crucial role in improving individuals' lives but also fosters continuous intellectual engagement through ongoing inquiries and challenges. With constant advancements and discoveries, medical students must remain active learners, experiencing both personal and academic growth. Specifically, neuroscience and neurology stand out as areas of particular interest due to the brain's complexity; despite being the organ responsible for regulating bodily functions and behavior, it remains the least understood, which positions it as a frontier of exploration. Similarly, technology presents a field of immense potential, which demonstrates humanity's ability to develop solutions to societal challenges. Given the numerous unresolved questions within medicine, technological advancements can offer insights for potential solutions, as exemplified by the CRISPR-Cas9 tool. The integration of these two fields can drive significant progress in both scientific research and medical practice.

The objective of this research was to evaluate the effectiveness of CRISPR-Cas9 in treating or potentially curing Huntington's Disease (HD). This gene-editing technology has the capacity to target and modify the CAG repeat sequence in the HTT gene, which is responsible for the disorder. Nonetheless, it remains uncertain whether this approach can effectively regulate the symptoms of HD, provide only temporary relief, or lead to a complete cure. This study seeks to address this unresolved question and critically assess the implications of implementing

CRISPR-Cas9 treatments in medical institutions worldwide. Additionally, it's essential to consider the patient's experience, overall well-being, and both the short- and long-term effects of the treatment to determine its feasibility for clinical application.

Therefore, the study provided further research and reassurance about the CRISPR-Cas9 treatment option for HD, underscoring areas of the procedure that must be improved for the safety of the patients and the effectiveness of the therapy overall. By publishing this project digitally, it will serve as a reference for current or future researchers or doctors that are working on this topic. Additionally, the paper presents an investigation aimed to improve HD patients' quality of life by ameliorating the symptoms of the disease, which make the disorder so devastating. Finally, the project is relevant to society as a whole, as it highlights the potential of CRISPR-Cas9 to treat diseases that burden public health systems. Given the rapid advancements in gene-editing technologies, raising awareness about their implications and future applications remains essential.

Introduction

Locomotor and sleep disturbances, cognitive complications, weight loss, dementia, and psychiatric symptoms—these difficulties characterize Huntington's Disease, a neurodegenerative disorder caused by a genetic mutation that has concerned doctors and scientific researchers lately. Its hereditary nature is alarming, since, according to the National Institute of Neurological Disorders and Stroke in the US, the child of an affected parent has a 50% probability of inheriting the mutation (NIH, n.d.). There is an urgent need for research and support for affected individuals, given that there is currently a lack of treatments to stop or reverse the progression of the condition.

Huntington's Disease (HD) is caused by an autosomal dominant mutation present in the first exon of the Huntingtin (HTT) gene that encodes for the Huntingtin protein on chromosome 4 (at the chromosomal location 4p16.3). In healthy individuals, there are 16-20 cytosine-guanine-adenine (CAG) repeats in the HTT gene, but HD patients have more than 40 CAG repeats. The trinucleotide CAG codes for the amino acid glutamine, meaning that HD patients have an expanded polyglutamine stretch and an abnormally long HTT protein that ends up misfolding and forming protein aggregates (clusters) within neurons. These aggregates are responsible for affecting a wide variety of cellular and molecular processes: they lead to neuronal death in the striatum and cortex, which are specific areas of the brain. This neuronal loss is the cause of the motor, cognitive, and psychiatric symptoms of HD (Alkanli et al., 2023).

Nevertheless, there is a possible treatment or cure for HD that was further analyzed to obtain insights into its future progression. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) with CRISPR-associated protein 9 (Cas9) is a gene-editing technology that

allows scientists to make precise changes to an organism's DNA, such as adding, removing, or changing specific sequences of the genome. The CRISPR-Cas9 system was first identified in the late 1980s and early 1990s in bacteria and archaea, but its first experimental information was obtained in the late 2000s (Tianxiang Li et al., 2023). This mechanism consists of two main components: a guide RNA (gRNA) that directs the system to a specific DNA sequence and the Cas9 enzyme that acts like molecular scissors to cut DNA at a targeted location. CRISPR-Cas9 holds promise for Huntington's Disease; this tool can target the mutated HTT gene with the expanded CAG repeats and cut the affected individual's DNA at this specific site. CRISPR-Cas9 guides the cell to repair the gene and this potentially can reduce or eliminate the harmful effects of the mutation. The research project focused on analyzing this treatment option from a molecular biology perspective to determine its effectiveness and the viability of implementing it on a larger scale.

Problem Statement

What is the effectiveness of implementing CRISPR-Cas9 genetic modification as a treatment for Huntington's Disease?

This question laid the foundation of the research project by establishing its final goal, which was to determine if CRISPR-Cas9 is useful in treating Huntington's Disease. Recent studies have approached the use of CRISPR technology for HD to discover if this technological tool can ameliorate the symptoms of the disease or cure it completely. In both cases, CRISPR is a significant contribution to the treatment of the disease, but its level of effectiveness varies according to the short-term or long-term benefits of its application (more long-term benefits would imply higher effectiveness). The response to this question provided insights into potential applications of CRISPR in other genetic diseases as well. In addition, the answer to the question presented a critical step towards finding other similar treatments for Huntington's Disease, taking into account that there's still no cure for it and only symptomatic relief is offered currently.

This research question was general enough to allow different approaches to it while being specific enough to study this precise genome-editing technology in a targeted disease. For instance, the question allowed research on the efficiency of CRISPR-Cas9 technology by itself through the study of its step-by-step process and past applications in medical scenarios. Meanwhile, it enabled the study of Huntington's Disease by itself from both a molecular and symptomatic perspective. These two separate topics, each with different approaches, then combined for the specificity of the question, which involved applying the knowledge obtained from the procedures in each concept and connecting it.

Finally, this question represented an unresolved medical issue that the author is interested in working on during her professional career as a neuroscientist or neurologist. A future goal is to have a pragmatic experience, either *in vivo* or *in vitro*, where DNA can be manipulated through CRISPR-Cas9 technology to either comprehend better the mechanisms of neurological diseases or to treat them. Hence, working on this research question provided the author with useful knowledge and a theoretical preparation not only for her career in general but also for this specific goal.

Objectives

General:

Determine, from molecular biology and through theoretical and practical studies, if the CRISPR-Cas9 gene-editing method is effective to treat Huntington's Disease.

Specific:

- Investigate the molecular mechanisms underlying the development of Huntington's Disease and its symptoms.
- Comprehend the step-by-step procedure of CRISPR-Cas9 *in vivo* and *in vitro.*
- Connect from a molecular perspective the hereditary nature of Huntington's Disease to its possible cure by modifying the patient's DNA sequences.
- Conclude through the study of past applications of CRISPR and interviews with specialists if CRISPR-Cas9 can treat or cure Huntington's Disease.

Methodology

Introduction

The pragmatic section of the research project, which was qualitative, focused on evaluating the effectiveness of CRISPR-Cas9 as a treatment for Huntington's Disease (HD) with the opinions and experience of specialists in the areas of interest; that is, neurologists, neuroscientists, general doctors, biotechnologists, or molecular biologists. The main objective was to find either a correlation or incongruence between the molecular mechanisms and experimentations studied in the theoretical framework and the testimonies of people who have researched CRISPR-Cas9 or treated HD. This enabled the analysis of the viability of implementing this technique and the possibility of using it in hospitals. It provided insights into the current state of research on the technology and if it still requires further experimentation before using it on HD patients. It also allowed to analyze if a meaningful amount of doctors or researchers have had the opportunity to experiment with this, or if the technique is still in a precarious or rudimentary state. The main challenge that arose was the struggle to find specialists who have directly studied this topic since it's still in development. Specifically, it was challenging to find experts locally, because, even if they've studied CRISPR and HD, many haven't engaged in direct research about the use of CRISPR as a treatment for HD. Regardless, the interviewees that were selected had sufficient knowledge about the topics they were asked about, leading to reliable and supported answers.

Abstract

Huntington's Disease (HD) is a rare neurodegenerative disorder that has concerned the medical field and negatively impacted the quality of life of its patients. It is an autosomal

dominantly inherited disease that is caused by an extensive repeat of the trinucleotide CAG on the cytogenetic location 4p16.3 in the Huntingtin (HTT) gene. Even though there are treatments that ameliorate its symptoms, such as medications and psychological support, there is still no definitive cure for the disorder. CRISPR-Cas9, a commonly used genome-editing tool in biomedical research, holds promise to treat or cure HD, but its use as a treatment is still in the process of investigation. This research project analyzed the efficiency of implementing CRISPR-Cas9 as a treatment for HD disease on a larger scale in medical centers.

The project used a theoretical approach based on scientific papers and previous research to analyze the genetic and molecular processes underlying the development of HD, the step-by-step process and applications of CRISPR-Cas9, and the success of past *in vivo* and *in vitro* experimentations of CRISPR-Cas9 in HD models. Secondly, the paper used a pragmatic method focusing on semi structured interviews with specialists in neurology, genetics, and biotechnology. This study aimed to provide insights and contribute to the previous knowledge on the topic, to accomplish the future use of CRISPR-Cas9 technology for HD patients as a treatment or cure.

Extended Methodology

The research project started with a theoretical investigation of the studies that have been done during the last few years about the topics of interest. These included papers about HD that mention the genetic and physiological processes that cause it, the clinical characteristics, symptoms, and current treatments for it. Additionally, articles about the procedure and mechanisms of CRISPR-Cas9 technology and how it has evolved throughout the years were studied. Finally, the theoretical section used studies that have tested CRISPR-Cas9 as a treatment

for HD through investigations *in vivo* and *in vitro,* such as in mouse models and cultured cells, respectively.

Next, the research project moved into a practical approach, specifically focused on interviewing 5 specialists who have worked on HD, CRISPR-Cas9, or both. The selected interviewees were mainly neurologists, workers in HD-specialized hospitals, neuroscientists, biotechnologists, and molecular biologists. Some of them were asked about the effect that HD has on its patients and the way it's being treated currently. Others were asked about the use of CRISPR-Cas9 in general terms and the exactitude or margin of error of the gene-editing technique. Finally, others were asked about the effectiveness of using CRISPR-Cas9 as a treatment for HD and the experimentation there has been of it up until today. These were semi-structured interviews—there were pre-established questions, but new questions that supported the responses to the pre-established ones were asked. Some interviews were done through text, while others were done through video calls, which lasted for approximately 1 hour each.

The questions for the interviews were divided into three groups: only HD, only CRISPR-Cas9, and CRISPR-Cas9 as a treatment for HD. The questions about HD were focused on the development of the disease, the symptoms, the current treatments, future cures, and the effect it has on the population. On the other hand, questions about CRISPR emphasized the effectiveness of the method, its margin of error, and the impact it has had on the overall population. Finally, questions about CRISPR as a treatment for HD focused on the effectiveness, risks, complexity, and current advancements in the treatment. Some specialists were asked questions only from one group, while others were asked from two groups, depending on their experience.

The following specialists were selected to answer the questions regarding HD development, symptoms, and effects on the quality of life of the patients. Dr. Diana Castellanos,

a neurologist who works as a physician and surgeon in Hospital de Caldas, located in Manizales, Caldas, Colombia, was chosen. She is a specialist in clinical neurology from Hospital Universitario Puerta de Hierro in Madrid, Spain. Additionally, Lisa Mooney, a social worker at UC Davis Huntington's Disease Clinic, was chosen. She is a licensed clinical social worker (LCSW) for the Huntington's Disease Society of America (HDSA) Center of Excellence and HDSA Northern California Chapter. Finally, Dr. Kyle Fink, the associate professor of the Neurology Department and the associate director of the Gene Therapy Center of UC Davis Huntington's Disease Clinic, was chosen. He received his Ph.D. in Neuroscience from Central Michigan University and the University of Nantes. UC Davis HD's Clinic, located in Davis, California, United States, is a center exclusively focused on working with HD patients, giving them support, and researching the disease, which is why it was the perfect fit for this set of questions. It also focuses on developing therapeutic options for neurological diseases by experimenting with patient-derived cell samples. There were some questions within the context of Colombia or the United States, so these were asked to Colombian and US interviewees, respectively (these will be indicated in the "Findings" section).

On the other hand, the following specialists were chosen to answer the group of questions focused on CRISPR-Cas9 effectiveness, applications, and error margin. Dr. Jhon Fredy Betancur, the research teacher of the medicine faculty at Universidad de Manizales, in Manizales, Caldas, Colombia, was chosen. He is licensed in biology and chemistry from Universidad de Caldas and a specialist in molecular biology and biotechnology from Universidad Tecnologica of Pereira, Colombia. Dr. Natalia Garcia, the genetics teacher of the faculty of medicine of Universidad de Caldas, was also chosen. She is a specialist in medical genetics and bioethics from Pontificia Universidad Javeriana, Bogota, Colombia. They both have experience

studying and working with CRISPR-Cas9 technology and other gene-editing techniques; therefore, they're a great fit for this set of questions.

Finally, the following specialists were selected to answer the questions about the effectiveness, viability, and potential of using CRISPR-Cas9 as a treatment for HD: Dr. Diana Castellanos, Dr. Kyle Fink, and Dr. Natalia Garcia. They all have a medical background, meaning that they know about HD, some being specialists in neurology, and they've also studied genetics and how gene-editing technologies can modify the genome as a means to treat genetic diseases.

Hypothesis

Huntington's Disease (HD) is a rare inherited neurodegenerative disorder that causes motor, psychiatric, and cognitive difficulties in its patients. Even though there are drugs and psychological support offered as treatments, there is still no cure for the disease. In addition, the disease progresses into levels of higher severity throughout the life of the patient, not only conditioning his/her life to the illness but also conditioning his/her family members. This makes HD a concerning disease that must be treated with urgency, favoring the public health system and the overall well-being of the worldwide population.

CRISPR-Cas9, on the other hand, is a gene-editing tool that was discovered in the 1980s, but it still needs to be investigated for further clarity. It allows DNA to be cut at a target site and repaired through homology-directed repair (HDR) or non-homologous end joining (NHEJ). Experiments so far have shown that CRISPR is a very efficient and precise method of gene editing that has a bright future in medical centers. Through applications of the technology in both animal samples and human cells, researchers have evaluated the strengths and weaknesses of CRISPR and worked on them to refine this powerful tool.

The genetic nature of HD enables it to be treated through CRISPR-Cas9 since this tool may be able to cut the gene that is causing the disease (mHTT). CRISPR-Cas9 interventions for HD have been evaluated in animals like mouse models, which have shown that CRISPR can decrease expression levels of mHTT and ameliorate the symptoms of the disease. However, there is still doubt on whether CRISPR can work as a treatment or cure for HD in humans.

This research project is focused on investigating the advancements in CRISPR-Cas9 as a treatment for HD and evaluating the usefulness of its application for HD patients. Taking into

account the current knowledge of the topic, the hypothesis is the following: CRISPR-Cas9 can be an effective treatment and cure for HD, but it still requires further investigations and refinements before its application in a clinical context.

Background

As a modern biotechnological advancement, CRISPR-Cas9 treatment for Huntington's Disease must be studied thoroughly before its application in medical centers on a larger scale. This section of the research project will focus initially on investigating the background information of HD, such as its etiology, epidemiology, inheritance, and symptoms. Then, the current treatments for HD, not including CRISPR-Cas9, will be explored. Subsequently, CRISPR-Cas9 technology will be studied, specifically the history of its discovery, its mechanism of action, applications, advantages, and challenges. Finally, applications of CRISPR-Cas9 to HD in living beings will be inquired.

Huntington's Disease: A Clinical Review

To later evaluate the effectiveness of CRISPR-Cas9 treatment in HD, it's crucial to have a general knowledge of the neurodegenerative disease by itself. Specifically, it's important to comprehend the molecular mechanisms underlying the condition, its symptoms, and the reason why current treatments haven't cured the disease completely. Roos, R. A. (2010) Huntington's disease: a clinical review from the *Orphanet Journal of Rare Diseases* is a medical review that provides information about HD's epidemiology, symptoms, etiology, diagnosis, genetics, and current treatments.

History and Epidemiology

The disease was first described by Waters, a patient with chorea (a symptom that causes involuntary movements of the limbs or facial muscles) in 1842. The condition was named Huntington's chorea in 1872 by George Huntington, who studied and described the disease. It is passed on genetically through an autosomal dominant mutation and usually has an onset in

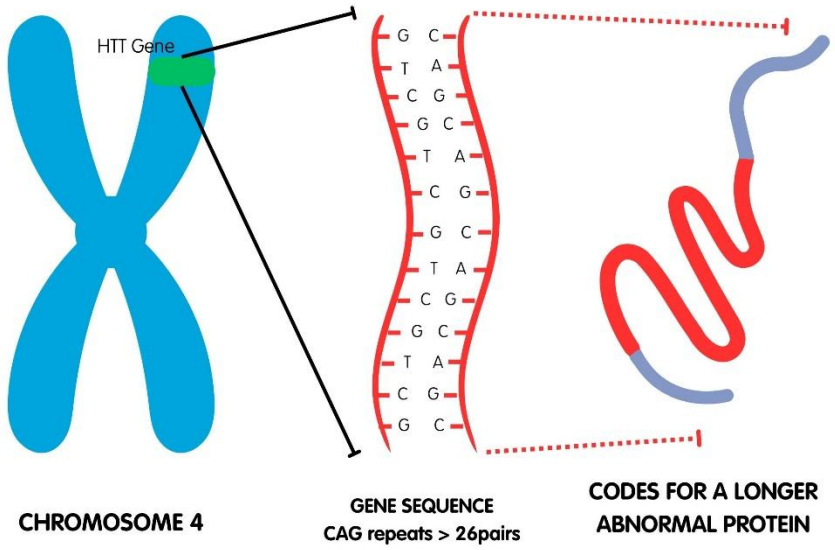
middle age. Specialists discovered, however, that the disease isn't only characterized by choreatic movements, but it also causes behavioral and psychiatric symptoms and dementia. In the 1980s, with full awareness of the extensive symptoms of the illness, the name was changed to Huntinton's Disease (HD). HD is a rare neuropsychiatric disorder that shows up in 5-10 out of 100,000 people in the Caucasian population. On the other hand, it shows up in Japan in a prevalence of one-tenth of the proportion of the Caucasian population (Roos, 2010). In Latin America, the overall pooled prevalence is 0.64 per 100,000 (Medina et al., 2024). These small rates among different populations and regions prove the rareness of HD in the world.

Etiology

The etiology of HD explains the set of causes at the molecular level that lead to the development of the condition. HD is an autosomal dominantly inherited disease that is caused by an extensive repeat of the trinucleotide CAG (cytosine, adenine, and guanine) on the cytogenetic location 4p16.3 in the Huntingtin (HTT) gene. The notation 4p16.3 refers to a chromosomal region in the short arm (p) of chromosome 4, specifically in region 16 and the sub-band 3 within region 16. The following figure displays the abnormal HTT gene and its location in the chromosome:

Figure 1

Abnormal Huntingtin Protein



Source: (Huntington's Victoria, 2024).

The HTT gene codes for the HTT protein and the CAG repeat is usually present on the first exon of the gene. Exons are regions of the genome that are present in the final mature mRNA transcript to code for amino acids. The CAG trinucleotide codes for the amino acid glutamine (Gln), and the long stretch of the CAG codon leads to an extended polyglutamine stretch in the HTT protein. (Roos, 2010). The amount of CAG repeats determines the development and the onset of HD:

> The wild-type contains a CAG repeat, coding for a polyglutamine stretch in the protein at that site in the range 6 to 26. Huntington’s disease is associated with 36 repeats or more. Definite clinical manifestation will occur if the number of repeats exceeds 40. The range. 36-39 leads to an incomplete penetrance of the disease or to a very late onset. The range

between 29 and 35, the so-called intermediate alleles, is unstable, which means that these alleles are prone to changes during reproduction. (Roos, 2010).

Huntington's Disease is usually caused when there are 36 or more CAG repeats, but a definite diagnosis is ensured when the CAG repeats are more than 40. Healthy individuals have between 6 to 26 CAG repeats, and the interval between 29 and 35 repeats, which separates the wild-type gene from the mutated gene, can change during reproduction (Roos, 2010).

Specialists have discovered an inverse correlation between the length of the CAG repeat and the age of onset of the disease, which means that individuals with a longer stretch (usually exceeding 55 repeats) develop the disease before the age of 20 years, also known as Juvenile Huntington's Disease (JHD) (Roos, 2010). As the number of repeats decreases, the onset of the disease takes place at an older age. Another pattern that has been observed is a positive correlation between longer CAG repeats and quicker weight loss during the disease. These patterns evidence the connection between the length of the CAG repeats and the development of HD symptoms.

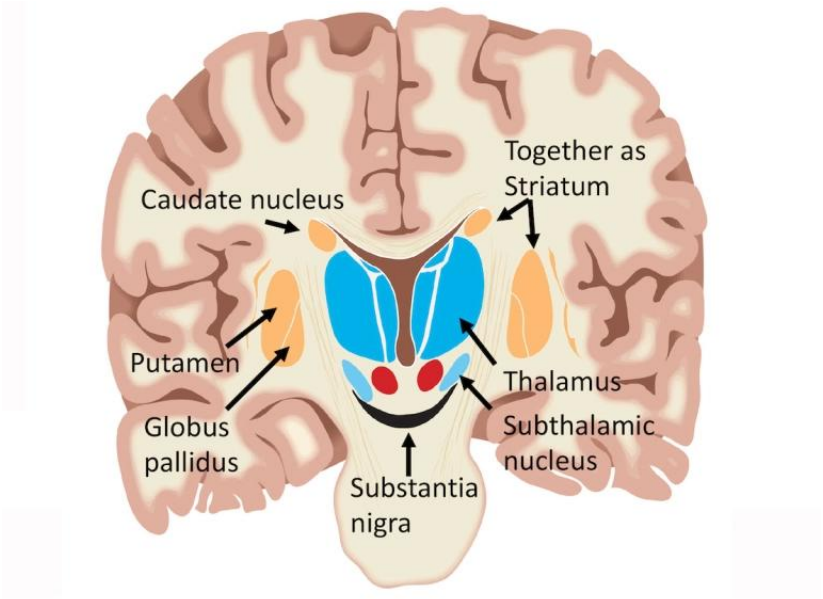
The wild-type HTT protein is important in synaptic function, which refers to the transmission of nerve impulses between two neurons or between a neuron and another target cell. It also has an anti-apoptotic function as it prevents controlled cell death, in this case of neurons. Finally, the wild-type HTT protein is protective against the toxic HTT mutant, which causes Huntington's Disease.

The mutated HTT protein leads to atrophy (decrease in size) of the brain, particularly in the striatum and the basal ganglia (Roos, 2010). The basal ganglia is a cluster of nuclei found in the neocortex of the brain, which is a region that comprises the largest part of the cerebral cortex and is in charge of higher-order brain functions. The function of the basal ganglia is to control

proprioceptive and conscious movements. On the other hand, the striatum is a cluster of interconnected nuclei located inside the basal ganglia, and its role is also related to movement control, as well as more complex cognitive tasks, such as decision-making, social interactions, and reward processing. The following figure shows the location of the basal ganglia and the striatum within the brain:

Figure 2

Basal Ganglia and Striatum



Note: The whole region shown is the basal ganglia, and the striatum is the yellow region indicated by the arrows.

Source: (Church, 2020).

This atrophy of the striatum, as well as apoptotic neuronal cell death and synaptic failure, which are caused by the absence of the wild-type HTT gene, lead to the development of chorea and the psychiatric and cognitive symptoms of HD (Roos, 2010).

Clinical Description

The motor symptoms initially occur in small facial muscles and distal extremities like the fingers and toes, but these unwanted movements extend gradually to more proximal and axial areas, such as the legs, arms, neck, and back. The most constant choreatic movement is the extension of the long back muscles. Additionally, continuous choreatic movements in the face can lead to a constant motion where an eyebrow is lifted, an eye is closed, the tongue is protruded with the lips pouting, and the head is bent or turned. Chorea causes struggles in talking and swallowing, leading to choking and the possibility of muteness development. It's also the source of unstable walking during daily life, making the person look as if he/she is moderately drunk (Roos, 2010).

Patients develop dysarthria, which takes place when the muscles used for speech are weak or hard to control, and dysphagia, which causes difficulty in swallowing. Other specific motor dysfunctions characterize HD: "All patients develop hypokinesia, akinesia, and rigidity leading to a slower pace of all activities (bradykinesia: slowness of movement) and a severe hesitation in embarking on a movement (akinesia: difficulty in starting movemevents)." (Roos, 2010). The combination of these motor difficulties with an increased muscle tone ultimately leads to the development of dystonia, which causes involuntary muscle contractions, and torticollis, which arises when a person's head tilts to one side due to tight muscles on one side of the neck.

Psychiatric symptoms, in contrast, are often present before the onset of motor symptoms. The most common sign is depression, which can be difficult to identify because the symptoms of depression, specifically weight loss, apathy, and inactivity, are also symptoms of HD. Patients also develop low self-esteem and anxiety. It has been observed that suicide among HD patients is

more frequent in early symptomatic individuals and premanifest gene carriers. Other psychiatric struggles include obsessions, compulsions, irritability, aggression, loss of interest, hyper-sexuality in the early stages, and hypo-sexuality in the later stages (Roos, 2010).

Furthermore, dementia or cognitive decline is another sign of HD and can be present long before the first motor symptoms. Patients with HD lose the capacity to distinguish what is relevant and what can be ignored, and they also have difficulty making mental adjustments or having mental flexibility. They cannot organize their lives or plan things as they did before, either. Other cognitive failures include memory damage, even though semantic memory (long-term memory of words, concepts, or numbers) can be conserved to a certain extent (Roos, 2010).

There are also secondary symptoms and signs related to HD. One of them is weight loss, which surprisingly isn't correlated to chorea or other movement disorders; instead, it has been observed that weight loss is positively correlated to the length of the CAG repeat, as well as decreased appetite and difficulty swallowing and handling food. HD patients also experience disturbances in their sleeping schedules and circadian rhythm (24-hour cycles that the body follows internally). Finally, autonomic disturbances, which occur when the autonomic nervous system does not regulate properly, might result in attacks of profuse sweating (Roos, 2010).

All of these motor, psychiatric, cognitive, and secondary symptoms cause difficulty in the life of the patient due to their progressive debilitating nature. Together, they make it hard for individuals with HD to care for themselves, maintain relationships, or live independently, requiring constant care or support as the disease progresses.

Development of the Disease

Huntington's Disease develops in three stages: at risk, preclinical (A), and clinical (B). In the at-risk stage, doctors determine if the person has the elongated CAG sequence in the chromosomal region 4p16.3. If the person does have the mutated HTT gene, then he/she will move into the preclinical and clinical stages until the end. Both the pre-clinical and clinical stages are divided into three parts, and, as the patient moves on through these sub-stages, he/she has less independence and requires increased care (Roos, 2010). The following table conveys the pre-clinical and clinical stages of a HD patient, including the parts of each stage:

Table 1

Stages during the Life of a Huntington's Disease Patient

A. Preclinical stage	
A1. At-risk stage (50%), one affected parent	- Anxiousness for the future
	- Uncertainty about carriership
	- Care for affected parent
A2. Gene carrier, premanifest stage	- Certainty about carriership
	- New position in the family
	- Renewed uncertainty about onset
	- Care for affected parent and own family
A3. Transition phase	- Strong feelings about changes in cognition

	- Changes in behaviour
	- Changes in motor activity
	- Uncertainty remains
B. Clinical Stage	
B1. Clinical stage I	- Presentation of first symptoms: neurological, cognitive, or psychiatric
	- Chorea most prominent symptom
	- Independent in ADL
	- Burden for the family mainly psychological
	- Rare death, unless suicide
B2. Clinical stage II	- Motor disturbance more generalized
	- Physical dependence starts
	- Burden for family psychological and physical
	- Death by other cause, suicide, euthanasia
B3. Clinical stage III	- Severe generalized motor disturbance
	- Almost complete physical dependence
	- Patient completely dependent on care
	- Burden for family mainly physical

	- Death

Source: (Roos, 2010).

Once the individual is diagnosed with HD, these are the stages that he/she follows before death. Over the last 20 years, it has become clear that psychiatric and cognitive changes are signs that appear many years before motor dysfunctions become visible. Many patients manifest that they undergo a gradual change in performance and behavior at work, and they also declare that they stayed home for some time after experiencing a 'depression' or a 'burnout' (Roos, 2010).

Management Including Treatment

Even though there's still no definite cure for HD, there are many therapeutic options available to treat the symptoms and to improve the quality of life of the patients. Chorea or hyperkinesia is treated with drugs like typical or atypical neuroleptics (dopamine receptor blocking) and tetrabenazine (dopamine depleting). Since excess dopamine (a neurotransmitter) and extra sensitive receptors cause involuntary movements, blocking these receptors or reducing dopamine levels through drugs is effective in regulating chorea. Neuroleptics are medications used for the treatment and management of symptoms related to psychosis. Two options of neuroleptics that have an antichoreatic effect are clozapine and olanzapine, but olanzapine is more practical because clozapine requires white cell control in the blood, which makes its treatment more complex. However, only tetrabenazine, the dopamine-depleting drug, has been shown to reduce chorea significantly. Another difficulty is that, to date, there's still no drug treatment available for hypokinesia, neuroprotection, or disease-delaying:

> Drug treatment for hypokinesia has been tried using antiparkinsonian drugs, but almost always with very disappointing results. In practice, therefore, dopaminergic drug are not prescribed. To date, despite several claims, no drug is available with any neuroprotective or disease-delaying effect. Disease modifying drugs are developed, but not available. Also embryonic cell implants, still under study, are not proven treatment options at the moment. (Roos, 2010).

This lack of medication to treat other symptoms related to motor dysfunction evidences the current incomplete treatment for HD and the need to develop more effective therapies for the disease.

In addition to medication for motor disturbances, there is also treatment available for depression and aggressive behavior. “Non-medical interventions available are: physiotherapy, occupational therapy, speech therapy, dietician, psychologist, social worker, and nurse.” (Roos, 2010). As the disease progresses, the dependency of the patient on a nurse increases, since he/ she eventually will need 24-hour care. The caregiver may have to deal with increasing responsibilities and require psychological help as well. Conversely, there is also medication available to regulate depression and aggression, including citalopram, fluoxetine, mirtazapine, valproinezuur, and carbamazepine (Roos, 2010).

Future Perspectives

Huntington’s Disease is devastating physically, psychologically, and socially, and, even though knowledge about the disease has increased significantly over the last two decades, there’s still no definite cure for it. HD is lifelong for both the individual and the family, which makes it even more tough to deal with it. HD also has complex molecular mechanisms that are difficult to

investigate and comprehend, even by expert researchers. Since 1993, the interest in HD has increased, which is proved by the increasing number of publications on the topic. Basic studies are focused on a better understanding of the pathophysiology of HD, which will lead to drug development that interferes with the pathological process of the illness. Other studies are focused on finding biomarkers, which are biological molecules found in the blood, other body fluids, or tissues that can be a sign of HD. “The developments are promising, but one thing is certain: the road to the solution is a long one.” (Roos, 2010).

CRISPR/Cas9 Therapeutics: Progress and Prospects

After investigating the background information about Huntington’s Disease, it’s crucial to inquire into the CRISPR-Cas9 tool, which will be later analyzed as a potential treatment for HD. To study this gene-editing technology independently, this research project will utilize Li, T., Yang, Y., Qi, H., Cui, W., Zhang, L., Fu, X., He, X., Liu, M., Li, P. F., & Yu, T. (2023) CRISPR/Cas9 therapeutics: progress and prospects from *Signal Transduction and Targeted Therapy*. This is a paper from a scientific journal that summarizes successful examples of clinical trials of CRISPR-Cas9, describes possible problems related to the technology, and reviews current developments in three aspects: gene-editing type, delivery vector, and disease characteristics.

Gene editing is a technology that modifies the genome sequence of an individual to induce insertions, deletions, or base substitutions. To date, there have been three main gene-editing technologies: zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR/CRISPR-associated protein 9 or Cas9), which is the most widely used tool in actuality (Tianxiang Li et al.,

2023). ZFNs and TALENs use proteins to target DNA strands, while CRISPR-Cas9 directs Cas9 to a specified location in the genome, improving the efficiency of the technology.

The CRISPR-Cas9 system evolved naturally in bacteria and archaea as a defense mechanism against plasmid transfers and phage infections. Bacteria and archaea incorporate a segment of foreign DNA into their DNA at a CRISPR site, which is a specific region of the genome composed of spacers (short sequences of foreign DNA) located between repetitive sequences of the bacteria or archaea. If the same foreign organism invades again, the microbe will transcribe this region, producing a guide RNA (sgRNA). SgRNA will guide the Cas9 enzyme to cut the invading DNA and remove the viral sequences completely from the microbe's genome (Tianxiang Li et al., 2023).

For sgRNA to recognize the sequence that it must cut, there must be protospacer-adjacent motifs (PAMs), which are short DNA sequences that are essential for the Cas9 enzyme to identify target sequences. The most frequent PAM in many organisms is NGG (any nucleobase followed by two guanine nucleobases), and the widespread presence of this PAM facilitates the use of CRISPR in plants, animals, and humans (Tianxiang Li et al., 2023). The manipulation of the nucleotide sequence of sgRNA allows the precise targeting of any gene, this to correct or silence disease-causing mutations and treating cancers, cardiovascular diseases, sickle cell anemia, and neurodegenerative disorders.

The discovery of CRISPR was accidental, and it took place in 1987 when Nakata et al. discovered an unusual sequence in the *iap* gene of Escherichia coli (E. coli). On the 3' end of the gene, Nakata et.al identified a sequence composed of five highly homologous sequences, containing 29 nucleotides each, separated by 32 nucleotides between each of the homologous

sequences (Tianxiang Li et al., 2023). This pattern in the genome of bacteria and archaea was detected in the following decade, as well as different CRISPR-associated proteins (Cas):

> In 2002, Janson et al. provided a generalized summary of the specific repeats that have been identified, naming these repeats as a family and using the acronym CRISPR for clustered regularly interspaced short palindromic repeats. In addition, multiple CRISPR-associated proteins (Cas)-Cas1 to Cas4- have been revealed in previous studies. (Tianxiang Li et al., 2023).

It wasn't until the identification of the pattern of bacterial and archaeal sequences that specialists denominated them as CRISPR. The identification of multiple Cas proteins was also significant because it allowed scientists to discover that each Cas protein has a unique function in gene editing, even though Cas9 is the enzyme specifically in charge of binding to DNA and cutting the target sequence.

In 2005, Mojica et al. discovered that most of the spacer sequences were derived from exogenous DNA and that only a small fraction of the spacer sequences came from the genome of the host (Tianxiang Li et al., 2023). They also found that viruses were more likely to infect organisms without homologous spacer sequences, meaning that the microbes with the CRISPR pattern were less likely to be attacked. Two years later, Mojica et al. confirmed that CRISPR is involved in bacterial and archaeal resistance against infection by plasmid transfer or external phages.

In 2011, Siknys et al. transferred the first CRISPR gene sequence from *Streptococcus thermophilus* (bacterium) to *Escherichia coli* (bacterium), and the *E. coli* successfully resisted plasmid formation (Tianxiang Li et al., 2023). This was the first report that CRISPR functioned

in a non-host bacterium, suggesting that the CRISPR system can function as a defense mechanism even in organisms that are not their hosts.

In 2012, Charpentier and Doudna did an investigation *in vitro*, where they discovered the mechanisms by which CRISPR/Cas9 works (Tianxiang Li et al., 2023). They purified the Cas9 protein from *S. thermophilus* and *Streptococcus pyogenes*, and this enabled the cleavage of prokaryotic DNA, suggesting that Cas9 cuts the genome. Additionally, Charpentier and Doudna also noticed that the cleavage site of Cas9 is controlled by a PAM sequence and a seed sequence in CRISPR RNA (crRNA). A seed sequence is a spacer-derived sequence around 20 nucleotides long that is located in crRNA; crRNA is a small RNA fragment that guides Cas9 to the target sequence of the host. These specialists also discovered that, by changing a nucleotide in the seed sequence, the system can be a gene silencer in a wide variety of situations.

Zhang Feng et al. published a paper in 2013 where they started to apply CRISPR to medicine, agriculture, and other fields (Tianxiang Li et al., 2023). They used human-derived 293T cells, which are a variant of the HEK293 (Human Embryonic Kidney 293) cell line (a population of cells that grow and divide under controlled conditions in a laboratory). They integrated trans-activating crRNA (tracrRNA), pre-crRNA, host factor ribonuclease (RNase) III, Cas9 from *S. pyogenes*, and the respective promoters and two nuclear localization signals (NLSs). TracrRNA forms a complex with pre-crRNA that guides Cas9 to the target sequence. On the other hand, RNase III recognizes pre-crRNA and processes it into smaller crRNA sequences. RNase III also cleaves the tracrRNA-pre-crRNA complex, producing the mature crRNA. Finally, the promoters and NLSs regulate the timing of CRISPR-Cas9 activity and ensure that the CRISPR components reach the nucleus, respectively.

The experiment targeted 30 base pairs located before the PAM at the human empty spiracle homeobox 1 (EMX1) locus, which is a gene that codes for a homeobox transcription factor (a protein that binds to DNA and regulates transcription). Zhang Feng et al. discovered that, by including the components mentioned above in the system, the cleavage of EMX1 was accomplished. “This experiment targeted 30 base pairs located before the PAM at the human empty spiracle homeobox 1 (EMX1) locus, and the results showed that cleavage of EMX1 was achieved with the inclusion of at least spCas9, tracrRNA and pre-crRNA.” (Tianxiang Li et al., 2023).

During the same year, Church et al. published a paper where they created a single guide RNA (sgRNA) by fusing two important CRISPR components: crRNA and tracrRNA. The fusion of these two RNA molecules enabled a simplified process because it eliminated the need for two separate RNA molecules. The authors also reduced the crRNA sequence length to 20 base pairs (bp), which is a sufficient length to guide Cas9 to the right place of the genome (Tianxiang Li et al., 2023). This discovery was important because it simplified the overall process and proved that CRISPR can work in mammalian cells, allowing a broader use of this technology.

Another important investigation in 2013 was done by Qi et al., who discovered the transcriptional regulatory tool dead Cas9 (dCas9) (Tianxiang Li et al., 2023). Dead Cas9 doesn’t have the cleavage activity that wild-type Cas9 has, but it can be guided by sgRNA to a specific DNA sequence and act as a transcriptional repressor; it blocks the expression of the target gene without making a double-strand break.

Months after CRISPR-Cas9 was shown to function in mammalian cells, scientists were able to apply gene editing to mice, fruit flies, rats, and plants like rice and wheat. In December 2013, Wu et al. published a study where scientists used CRISPR-Cas9 to treat cataracts (a

condition where the eye's natural lens becomes cloudy) in a mouse model (Tianxiang Li et al., 2023). They coinjected the mRNA coding for Cas9 with an sgRNA into the fertilized eggs of the mice that would be born with cataracts. Of the 22 mice obtained, only 10 carried the mutant allele: 6 of them had NJEH-mediated insertions and deletions, while 4 had HDR-mediated repairs. All four HDR-mediated repaired mice were cured completely, while two of the six NJEH-mediated repaired mice were cured completely. These results indicated that CRISPR therapy could modify the genome and treat genetic diseases.

There was another study done by Schwank et al. during the same period, where they isolated intestinal stem cells from two patients with cystic fibrosis transmembrane conductor receptor (CFTR) mutation (Tianxiang Li et al., 2023). They were successful at correcting the disease-causing mutation using CRISPR-Cas9 technology.

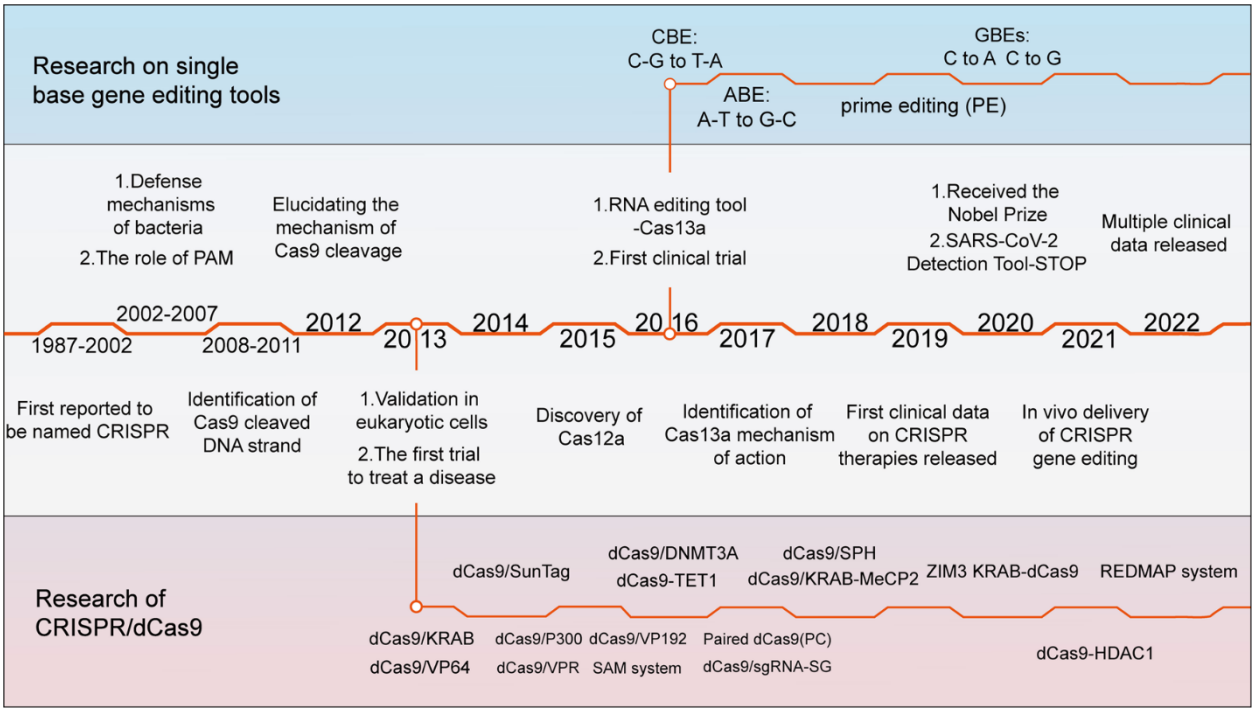
In 2016, Komor et al. argued that CRISPR should correct single bases instead of excising whole sequences and allowing random recombination (Tianxiang Li et al., 2023). They experimented with cytidine deaminase, which is an enzyme that can edit single bases: "Cytidine deaminase catalyzes the deamidation of cytosine into uracil, which subsequently changes back to thymine through replicative division. Thus, they integrated CRISPR/dCas9 with rat-derived cytidine deaminase (APOBEC1) and successfully achieved C to U base conversion." (Tianxiang Li et al., 2023). This successful base substitution set the basis for the subsequent invention of more base-substitution methods that made CRISPR more efficient. During the same year, scientists discovered Cas13a, which is another CRISPR-associated protein that can cleave specific RNA sequences, rather than DNA sequences as Cas9 does it. This discovery was important, as Cas13a has the potential to treat RNA-based diseases.

In October 2016, Lu and colleagues conducted the first clinical trial of CRISPR-Cas9 at West China Hospital in Sichuan, China (Tianxiang Li et al., 2023). Lu et al. obtained T cells (a type of white-blood cell called lymphocytes) from patients and introduced plasmids encoding Cas9 and sgRNA that targeted the PD-1 gene through electroporation. The PD-1 (Programmed Cell Death Protein 1) gene encodes a protein important in regulating the immune system by preventing the overactivation of T cells. On the other hand, electroporation is a method that consists of inserting DNA into a cell using quick electric pulses that generate temporary openings in the cell membrane. These CRISPR-Cas9-edited T cells were injected back into patients and the results showed a significant reduction in PD-1 expression in the gene-edited T cells, thus proving the feasibility of CRISPR-Cas9 therapy. Besides, follow-up studies of the patients after CRISPR-edited T-cell injections showed that they didn't experience significant adverse effects and that two of the patients were in stable condition, proving the safety of the method.

Studies of CRISPR-Cas9 were ongoing during the following years, leading to the release of the multiple clinical data on CRISPR therapies and other *in vivo* applications. In October 2020, French microbiologist Emmanuelle Charpentier and American biologist Jennifer Doudna were awarded the Nobel Prize in Chemistry for "developing a new approach to genome editing." (Tianxiang Li et al., 2023). Research on the topic is stipulated to continue during the following years, providing each time more advancements and clinical applications. The following timeline displays the most remarkable events of CRISPR-Cas9 discovery and advancements during the last few years:

Figure 3

Timeline of Major Events in the Development of CRISPR/Cas Technology and Representative Cas Variants



Note: The image above shows the progress in CRISPR technology research, going from its first report in 1987 to the release of multiple clinical data of its applications in 2022.

Source: (Tianxiang Li et al., 2023).

CRISPR has had a long road of investigations and discoveries, which have revealed that it is a very efficient technique to modify the genome according to the specific necessities of the individual. However, there is a long path to follow to implement CRISPR in clinical contexts in a standardized way, since there still has to be more certainty about the applications of the technique in humans. This will most probably be accomplished through investigations in cultured cells, animal models, and humans in the future years.

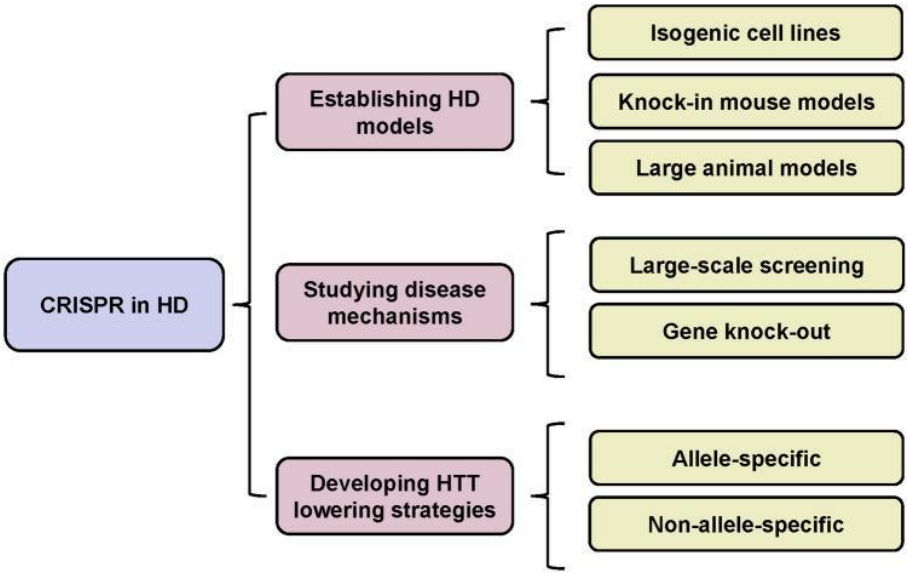
CRISPR-Based Genome-Editing Tools for Huntington's Disease Research and Therapy

Now that there's information about both Huntington's Disease and CRISPR-Cas9 independently, it's important to comprehend the mechanisms underlying the application of CRISPR to HD's research and treatment. For this purpose, the research project will utilize Qin, Y., Li, S., Li, X. J., & Yang, S. (2022) CRISPR-Based Genome-Editing Tools for Huntington's Disease Research and Therapy from *Neuroscience bulletin*. This paper offers a review of CRISPR-based genome-editing tools and their applications in HD research, this with the future goal of advancing genome-editing technologies for HD treatment.

Earlier gene-editing tools, such as ZFN as TALEN, have been used in HD research successfully. However, after it was showed that CRISPR is effective in mammalian cells, this new technology replaced ZFN and TALEN in HD research (Qin et al., 2022). The following figure displays the applications of CRISPR technology in HD research:

Figure 4

Summary of the Major Applications of CRISPR-Based Genome Editing in Huntington's Disease (HD) Research



Note: The table shows three different ways in which CRISPR-Cas9 has been utilized to advance in Huntington's Disease research during the last few years.

Source: (Qin et al., 2022).

One of the applications of CRISPR was to establish HD models, which replicate the disease to study its mechanisms and potential treatments. Three HD models that have been used are isogenic cell lines (genetically identical cell lines that differ only in a specific gene or mutation), knock-in mouse models (genetically engineered mice where a specific gene or mutation is inserted), and large animal models (applications of CRISPR in animals larger than mice). Another application of CRISPR is the study of the mechanisms of HD, usually through two methods: large-scale genetic screening (**s**tudy of the function of genes) and gene knock-out (removal of genes). Finally, CRISPR can also be applied to develop HTT lowering strategies

through allele-specific (precise and selective) and non-allele-specific (affects both copies of a gene) editing (Qin et al., 2022).

In 2014, CRISPR was first used to generate isogenic HD cellular models (Qin et al., 2022). Researchers used CRISPR to target exon 1 of the HTT gene and make a precise cut in the CAG expansion. To repair the damage caused by the cut, researchers induced a donor DNA construct (a piece of DNA used as a template) into the edited cells. This way, researchers created different versions of human induced pluripotent stem cells (iPSCs), which are cells that can differentiate into various cell types and are typically used in disease modeling. They created iPSCs with 21, 72, and 97 CAG repeats to study how different levels of CAG repeats affect cellular function and development of HD. Since then, other researchers have also generated isogenic HD human iPSCs through CRISPR and have reported other observations: "…phenotypic abnormalities in such cells, including impaired neuronal differentiation, increased sensitivity to growth factor withdrawal, mitochondrial defects, and gene expression changes." (Qin et al., 2022). The fact that these edited cells displayed the molecular effects of HD is evidence that this method of using CRISPR for HD research holds promise in the future.

Nevertheless, mouse models remain the principal platforms for HD research (Qin et al., 2022). CRISPR-Cas9 offers versatility in creating novel knock-in mouse models (mice with gene insertions) to test HD-related hypotheses. Researchers have proposed the toxic fragment hypothesis, which suggests that it's not the full-length mutant HTT protein that is toxic itself, but instead, the cleaved N-terminal HTT fragments are the ones causing neurodegeneration in HD (Qin et al., 2022). According to the hypothesis, the full-length mutant HTT protein undergoes proteolytic cleavage, which is a process where enzymes cut the protein into smaller fragments; particularly, there are N-terminal fragments resulting from those cuts, which are the portions of

the protein containing the expanded glutamine stretch. The N-terminus is located at the start of the amino acid chain, and it is characterized by containing an amino group (NH_2) that is not involved in a peptide bond (bond formed between amino acids). According to the hypothesis, these N-terminal fragments that result from proteolytic cleavage of the mutant HTT protein are responsible for neurotoxicity and death of neurons.

To evaluate this hypothesis, researchers used CRISPR-Cas9 to edit the HTT gene in embryos of HD140Q knock-in mice. They created two knock-in mouse lines expressing different N-terminal HTT fragments. These two lines contained a stable N-terminal mutant HTT fragment equivalent to exon 1 HTT, and they both also showed similar neuropathology and disease progression. These results suggested that exon 1 HTT is the key pathological form that causes Huntington's Disease (Qin et al., 2022).

Another hypothesis that has been evaluated in mice suggests that CAG expansion in the HTT gene leads to repeat-associated non-AUG (RAN) translation to produce toxic peptides (Qin et al., 2022). RAN translation is an unconventional protein translation that is common in regions of the genome with repetitive nucleotide sequences and which starts protein synthesis without the usual AUG start codon. Researchers tested this hypothesis using a knock-in mouse model, which they edited using CRISPR so that it expressed mutant HTT mRNA, but not the mutant HTT protein. However, researchers didn't perceive any significant role of RAN translation in the mice:

> Via CRISPR/Cas9-mediated genome editing, a knock-in mouse model that expresses mutant HTT mRNA but not mutant HTT protein was established. RAN-translated products were not detected in this mouse model, nor were HD-related pathological

> changes, indicating that RAN translation does not play a major role in HD pathogenesis. (Qin et al., 2022).

Since RAN wasn't important in HD development, the hypothesis was declared false.

Even though mice models have provided useful insights into HD development, they lack the neurodegeneration of the striatum, which is a key effect in HD patients. Therefore, in 2018, an HD knock-in pig model was established. Researchers used CRISPR-Cas9 and a donor vector (DNA molecule that transports DNA to target places) carrying human exon 1 with 150 CAG repeats to knock in mHTT in fetal pig fibroblasts (cells that contribute to connective tissue development). Subsequently, somatic cell nuclear transfer, which is a laboratory strategy to create an embryo from an egg cell and a body cell, was used to generate HD knock-in pig newborns. This pig showed neurodegeneration in the striatum and HD-like phenotypes, which evidenced the effectiveness of using large animals to investigate HD pathogenesis and potential therapeutics (Qin et al., 2022).

In the following years, CRISPR-Cas9 has been used and is intended to be utilized in the future to address two important issues regarding HD pathogenic mechanisms: "…first, how to efficiently and reliably perform screening and second, how to find the key targets that are most relevant to HD pathogenesis." (Qin et al., 2022). In 2020, researchers performed an unbiased genome-wide genetic screening in the mouse central nervous system by using CRISPR and short hairpin RNA (shRNA) lentivirus, which is a system that uses shRNA delivered by a lentiviral vector to silence gene expression (Qin et al., 2022). Researchers found that, in wild-type mice, neurons are vulnerable to perturbations of synaptic function, autophagy (consumption of the body's tissue), proteostasis, mRNA processing, and mitochondrial function. They also used

CRISPR and shRNA to identify the genes that are important for neuronal viability when mHTT is present:

> Screening results using one HD transgenic mouse model and one HD knock-in mouse model revealed that genes involved in methylation-dependent chromatin silencing, dopamine signaling, and members of the *Nme* gene family are genetic modifiers of mutant HTT toxicity, suggesting these genes could be new targets for therapeutic interventions. (Qin et al., 2022).

Recent research studies have revealed that there are new genes, including those from the *Nme* gene family, that are involved in the toxic effects of mHTT protein and the development of HD. Some of these genes are involved in regulation within the genome since they can silence other genes through methylation-dependent chromatin silencing and influence the progression of HD. Others are involved in regulating dopamine signaling, which is altered in HD.

This research continued through the manipulation of the expression level of individual genes to determine their effect on the development of Huntington's Disease. Some important genes that have been identified are *FAN1*, *RRM2B*, and *MLH1* (Qin et al., 2022). Through CRISPR, researchers generated knock-out mice for each gene (mice with the target gene removed from their genomes) and then crossed these mice with HD knock-in mice to observe the effects of these genes *in vivo*. They are involved in neuronal loss in the striatum and neurotoxicity, which are effects of HD, and this makes them important targets for in-depth investigations of HD.

This study presents a significant advancement in the investigation of CRISPR-Cas9 and its application in Huntington's Disease. It uses animal models and shows the success of

CRISPR-Cas9 in manipulating the genome and reducing the biological effects of the disorder. The results of these studies and experiments provide powerful insights to continue investigating this topic and to each time get closer to implementing CRISPR in human patients of HD.

Theoretical Framework

Implementing CRISPR-Cas9 as a treatment for Huntington's Disease is a relevant topic that provides compelling insights for the development of the medical field. As the demand for therapies increases daily, working on new treatments and technologies is crucial to address unmet medical needs, improve clinical services, and improve overall quality of life. Even though CRISPR-Cas9 was discovered in the nineteen eighties, it's still in the process of evaluation by researchers, who will determine if it can be applied as a treatment for Huntington's Disease on a large scale. As specialists analyze gene-editing technology, it's important to understand the theoretical context of how the technology works and the procedure it must follow to be successful. Additionally, it's essential to comprehend the molecular mechanisms underlying the development of HD, which will later be related to the effects that CRISPR-Cas9 treatment has on these biological processes. Therefore, the theoretical framework will focus on understanding and analyzing these procedures, to respond to the research question and the objectives that were established previously in the project.

This section will start by studying the molecular and physiological impacts of HD, ranging from cellular organelles to brain structures, as well as inquiring about the neuropsychiatric symptoms of the disorder. These effects of HD will be studied from the diagnosis to the progression of the disease, allowing an observation of how the condition develops throughout time.

Subsequently, the theoretical framework will investigate the highly specific and rigorous step-by-step procedure that is followed during CRISPR-Cas9 treatments, the structural characteristics of the proteins and other molecules involved, and the effectiveness of the tool. To accomplish this, current applications of CRISPR-Cas9 in the medical field will be studied, as

well as the purposes for which it is used, the organisms in which it has been applied, and the different methods to use it (*in vivo* and *in vitro*).

Next, the first and second sections of the theoretical framework will be interconnected: the research project will investigate the mechanisms by which CRISPR-Cas9 can be implemented for HD patients and the possible effectiveness or failure of this new treatment. This goal will be attained by the analysis of past and current studies on this topic, which usually involve CRISPR-Cas9 applications *in vivo* through studies in animals with HD, such as mouse models, and *in vitro* through investigations in cultured cells, which are usually mammalian cells. The results of these experiments will be used to observe patterns in their success or failure to evaluate the possible effectiveness of the procedure on humans in a clinical context.

Another important part of the theoretical framework will be investigating if there have been applications of CRISPR-Cas9 on human HD patients. In case this treatment hasn't been used in real scenarios, this will be evidence that there is still a need for more studies prior to this application, and it won't deny the future potential of the treatment if investigations are continued. On the other hand, in case the treatment has been used in real life, the results will serve as evidence to analyze the effects that CRISPR-Cas9 has on HD patients and the overall success of the treatment.

Finally, all the subsections of the theoretical framework will converge to conclude if applying this technology in clinical centers on a large scale and in the long term is a considerable option. CRISPR-Cas9 should become a standardized treatment for HD patients if it can treat the symptoms or cure the disease completely to improve the overall quality of life of the patient. In the final part of the theoretical framework, it's also crucial to make the distinction on whether the

treatment serves as a regulator of the motor, cognitive, and psychiatric symptoms of HD, or if it can completely cure the disease.

The methodologies that will be used in this section include a theoretical study of the topics of interest through scientific articles that explain the functioning of CRISPR-Cas9 and HD. Following that, practical applications of the technology for the disease will be investigated. Finally, the theoretical explanations will be connected to the practical contexts to analyze the procedure rigorously and determine its overall success.

Clinical Characteristics of Huntington's Disease: Neuropathology and Neuropsychiatry

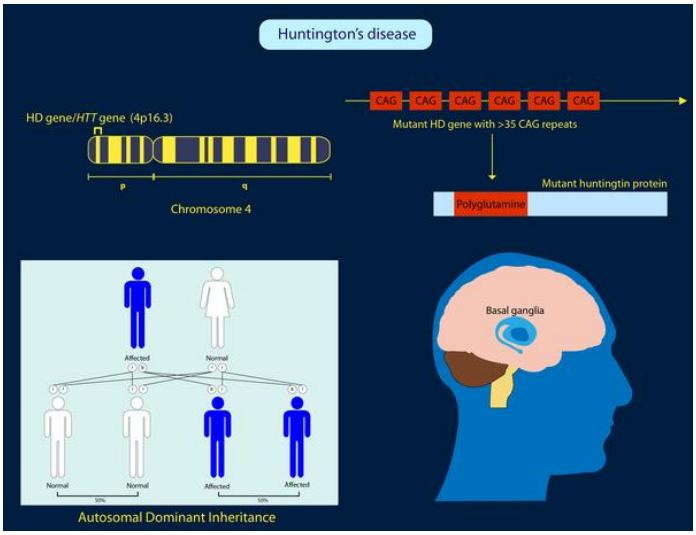
Huntington's Disease (HD) is a rare neurodegenerative disorder that has progressive motor, cognitive, and psychiatric disturbances. The median survival period for its patients is 15 to 18 years after onset (Caron et al., 2020), and there is still no definite cure for the disease, which makes it a concerning subject for the medical field. While doctors seek to work on current medical systems to improve patient quality of life and services, there are conditions like HD that must be addressed urgently because of the negative effect they have on the overall population. Addressing and investigating HD involves understanding the molecular and physiological failures that take place during its development, as well as comprehending how the patient's lifestyle is affected. This section of the theoretical framework will focus on explaining the neuropathology of HD, which studies the structural changes of the nervous system that take place during the disease, and the neuropsychiatry of HD, which refers to the mental or emotional disturbances that are related to the disordered brain function.

Pathological Mechanisms of HD

Huntington's Disease is caused by the expansion of the CAG codon in the first exon of the HTT gene, which encodes the glutamine amino acid. This stretch of amino acids, also known as the polyQ stretch, is located at the N-terminus of the HTT protein. Glutamine amino acids then form the glutamine neurotransmitter, and it has the function of sending signals in the brain, especially those related to chorea (movement). Glutamate, the precursor of the glutamine amino acid, is produced in the muscles, lungs, and brain, showing the relationship between this neurotransmitter and the control of essential functions of the body, such as movement. Since the CAG codon is expanded, so does the amino acid chain, forming a poly-glutamine expansion that results in the formation of protein aggregates (proteins that are misfolded). Aggregates in the brain lead to many of the physiological responses of HD, including mitochondrial dysfunction, apoptosis, excitotoxicity, and transcriptional dysregulation (Serdar et al., 2022). The following figure displays the mutant HTT gene and its respective location on the chromosomal section 4p16.3, the structure of the brain that it affects (basal ganglia), and how it is inherited (autosomal dominant mutation):

Figure 5

mHTT Gene Location and Autosomal Dominant Inheritance



Source: (Martin, n.d.)**.**

The wild-type HTT (the normal gene) codes for a scaffolding protein that "helps coordinate other proteins and cellular functions" (Serdar et al., 2022). It is important in transcriptional regulation and the production of brain-derived neurotrophic factor (BDNF), which is an important factor for the survival of striatal (from the striatum) and cortical (from the cortex) neurons. Wild-type HTT also has other vital functions, such as regulating axonal transport, exchanging endosomes, and recycling organelles and vesicles. When the wild-type HTT is mutated in HD, the following pathological mechanisms develop: "mHTT causes disease through a dominant toxic function gain mechanism. These pathological mechanisms include mechanisms such as early transcription dysregulation, synaptic dysfunction, proteasome dysfunction, aggregate pathology, oxidative damage, mitochondrial dysfunction and extrasynaptic excitotoxicity" (Serdar et al., 2022). Since HD dysregulates mechanisms that are essential for the functioning of the brain, it leads to the development of the severe motor,

psychiatric, and cognitive symptoms that characterize HD. The dysfunctions mentioned previously will be explained in the following.

Early transcription dysregulation refers to the disruptions in the normal process of transcription that occur during the initial stages of gene expression. Since the HTT protein during HD is mutated, it disrupts transcription factors and other proteins important in normal transcriptional regulation. Another effect of HD is synaptic dysfunction, which refers to abnormalities in the way neurons communicate at synapses, the structures where neurons send signals to each other. Since mHTT disrupts the functions of key proteins involved in synaptic transmission, in addition to producing excess glutamate (neurotransmitter), it affects communication between neurons, particularly in the striatum.

On the other hand, proteasome dysfunction during HD refers to failure in the proteasome system, a complex that degrades or recycles damaged or misfolded proteins. Since mHTT leads to the formation of protein aggregates with an abnormal structure (aggregate pathology), the proteasome struggles to recognize and degrade them, causing their accumulation in the striatum. Additionally, HD causes oxidative damage because it leads to the formation of unstable molecules containing oxygen that damage DNA, proteins, and lipids.

Mitochondrial dysfunction is another characteristic of HD, as mHTT causes a lack of ATP production when it disrupts the electron transport chain during cellular respiration, as well as alters the mitochondrial release of calcium ions to maintain the mitochondrial membrane potential. Finally, extrasynaptic excitotoxicity is an effect of HD where the excessive stimulation of glutamate receptors causes neuronal damage and death.

Now that the general pathological mechanisms of HD have been explained, it is important to deepen the topic of neurodegeneration, as it is one of the main effects of HD. Neurodegeneration is the main cause of the motor, psychiatric, and cognitive symptoms of HD. Therefore, analyzing it can provide useful insights to find treatments or cures for the disorder.

Mechanisms of Neuronal Degeneration in HD

The principal neuropathological effect of HD is the degeneration of neurons, which is preferential for enkephalin-containing, medium-spiny neurons of the indirect pathway of movement control located in the basal ganglia (Caron et al., 2020). The characteristics of these specific neuron type and their location in the brain will be explained.

The neurons that are degenerated during HD are encephalin-containing neurons (Caron et al., 2020). Enkephalins are small peptides that belong to a class of compounds called endogenous opioids. They are part of this group because they are produced naturally in the body and have opioid-like effects (these include sensations like pain relief and euphoria). Enkephalins bind to opioid receptors in the brain and the spinal cord, leading to induced feelings of euphoria and relaxation, as well as reduced pain perception. In this case, enkephalin-containing neurons are those that produce enkephalins and release them to opioid receptors located in other neurons (Olmos, n.d.).

In addition, the neurons affected in HD are classified as medium spiny neurons (MSNs), which are a type of neuron found primarily in the striatum (Caron et al., 2020). Their main characteristic is their high density of dendritic spines, which are small protrusions on their dendrites (locations where they receive inputs from other neurons). MSNs are essential for coordinating movement, reward processing, and other aspects of learning and decision-making.

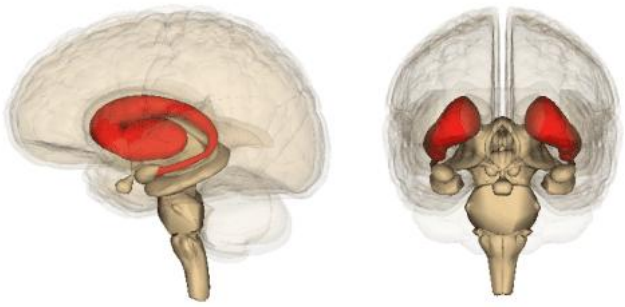
Moreover, the basal ganglia are the locations where these neurons are found. They are a group of brain structures linked together, and they are best known for controlling movement, learning, emotional processing, and other crucial functions. It is specifically a cluster of nuclei found deep in the neocortex of the brain, which is the outermost region of the brain that is involved in high-order brain functions like sensory perception, motor commands, and reasoning (Young CB et al., 2023). The basal ganglia contain different parts in its largest component, which is the corpus striatum:

> The largest component of the basal ganglia is the corpus striatum which contains the caudate and lenticular nuclei (the putamen, globus pallidus externus, and internus), the subthalamic nucleus (STN), and the substantia nigra (SN). These structures intricately synapse onto one another to promote or antagonize movement (Young CB et al., 2023).

The striatum is the main component that is affected during HD because it's the area that experiences the most neuronal degeneration. Inside the striatum, there is evidence for the loss of neurons in the globus pallidus, subthalamic nucleus, and substantia nigra (Caron et al., 2020). Additionally, the neurons that are degenerated in these parts of the basal ganglia go through the indirect pathway of movement control, which is a type of circuit that inhibits unwanted or excessive motor activity. The following figure displays the location of the corpus striatum in the human brain, which is the location where the neurodegeneration described takes place:

Figure 6

Corpus Striatum



Source: (Bonifacio, 2019)**.**

The degeneration of the neurons that contain the characteristics mentioned previously (enkephalin-containing, medium spiny neurons, and the indirect pathway of movement control) leads to an overall disruption of the movement control and cognition pathways and leads to the development of the evident symptoms of HD (Caron et al., 2020).

Now that the type of neurons that degenerate during HD is clear, it's important to understand the mechanisms by which these cells die. For this purpose, the scientific article by Sawa, A., Tomoda, T., and Bae, B.I. (2003) called Mechanisms of neuronal cell death in Huntington's disease from *Cytogenetic and Genome Research* will be studied and analyzed. This text explains the mechanisms through which the mutant HTT gene causes cytotoxicity (cellular damage) and neuronal cell death in HD brains.

Neurodegeneration in HD is initially caused by the expansion of the glutamine stretch in the HTT gene, but the normal stretch of the HTT gene has several important roles, such as endocytosis (the process by which substances are brought into the cell) and the upregulation of

brain-derived neurotrophic factor (BDNF), which is an important protein for survival. Normal HTT also protects the cells from apoptosis, and this has been confirmed by investigations where the inactivation of the HTT gene in mouse models has led to progressive neurodegeneration (Sawa et al., 2003).

Overexpression of mHTT kills many types of cell lines and increases cellular susceptibility to death (Sawa et al., 2003). The cells that die because of mHTT usually manifest several apoptotic features, such as nuclear changes and caspase-3 activation (an important enzyme in apoptosis). Other apoptotic features present include nuclear condensation and fragmentation, susceptibility to mitochondrial membrane depolarization (making the cell more positive than its resting state), and DNA ladder formation, which is the cleavage of DNA into fragments. Finally, overexpression leads to HTT accumulation in cytoplasmic vacuoles that are larger than normal and look similar to autophagosomes (structures important for autophagy, a process where cells degrade their components) (Sawa et al., 2003). The enlargement of vacuoles can be associated with apoptosis because, as part of the degradation and recycling of cellular components, vacuoles may swell.

Currently, there are several drug-induced and genetically engineered animals as models of HD, including mainly mice and rats. Transgenic mice overexpressing exon 1 of the HTT gene have revealed dying neurons with neuronal intranuclear inclusions, which are abnormal protein aggregates that form within the nuclei of neurons (Sawa et al., 2003). Neurons in these mice displayed the condensation of both the cytoplasm and the nucleus, ruffling of the plasma membrane (formation of protrusions), and mitochondrial failure, specifically a deficit of energy metabolism in the brain (Sawa et al., 2003).

Other investigations have included the brains of HD patients. These have revealed bilateral, symmetric atrophy of the striatum in 95% of HD brains (Sawa et al., 2003). It has also been displayed that, in later stages of the disease, the atrophy of the cerebral cortex becomes pronounced, which increases neuronal degeneration as HD develops.

The mechanisms through which neurodegeneration takes place, the specific locations of the brain where it takes place, and its relationship with HD development must be understood before looking for effective treatments for the disease, such as CRISPR-Cas9. Investigations of HD must take into account the molecular effects that were mentioned previously to find patterns and evaluate if tentative treatments can ameliorate them. Now that neurodegeneration in HD has been studied, it's important to investigate how these physiological impacts reflect in the psychiatric symptoms of the disorder.

Neuropsychiatry of HD

The molecular processes that are triggered during HD cause psychiatric disorders in patients, such as depression, mania, Obsessive Compulsive Disorder (OCD), executive dysfunction syndrome, apathy, and irritability. In the following subsection of the theoretical framework, these psychiatric effects will be described and explained.

Depression is a common neuropsychiatric disorder that develops in HD patients. A depressed mood is observed in approximately 35% to 60% of the patients. It has also been observed that HD patients have a suicide rate 4 to 6 times higher than that of the general population (Rosenblatt, 2007). There are features of HD that might lead to the underdiagnosis or overdiagnosis of major depression. For example, weight loss and apathy, which are common symptoms in HD patients, might be seen as indicators of depression, or they might be dismissed

as the patient's reaction to having HD. Depression in HD is also associated with hypometabolism of glucose in the orbitofrontal and inferior prefrontal regions of the brain. This is consistent with the fact that depressed patients without a primary neurologic disorder present a lack of metabolism in the prefrontal cortex (Rosenblatt, 2007). It has been said that major depression can precede the movement disorder in HD by years, but major depression is a common condition in the population, so it should not be considered automatically as an indicator for HD genetic testing or HD diagnosis (Rosenblatt, 2007).

Other psychiatric disorders that have been perceived in HD patients are mania and bipolar syndromes, which have a lifetime prevalence of 5% to 10% in HD patients (Rosenblatt, 2007). Some symptoms of these disorders include an irritable mood, impulsiveness, hypersexuality, a decreased need for sleep, a grandiose self-attitude, and delusions and hallucinations. As with depression, mania can present early in the disease, but it cannot be considered immediately as an indicator of HD because its symptoms can also be part of the patient's reaction to having HD. A classic presentation of mania usually includes three main elements: an irritated mood, a paranoid thought content, and symptoms of overactivity (racing thoughts, pressured speech, hypersexuality, and a decreased need for sleep). When diagnosed, mania is usually treated with a mood-stabilizing agent, "usually an anticonvulsant such as divalproex sodium or a neuroleptic" (Rosenblatt, 2007).

Obsessive Compulsive Disorder (OCD) has also been present in HD patients, and it's a condition that features unwanted thoughts, features, or obsessions. Some behaviors in HD patients that indicate OCD are repetitive movements and speech, perseveration, inflexibility, and preoccupation with idiosyncratic topics. Some drugs that are useful in treating OCD are serotonergic agents, which are compounds that regulate the neurotransmitter serotonin, but it's

recommended that OCD patients also go through a behavioral treatment. This includes distraction, the settlement of a routine, and the management of the expectations of friends and family through education (Rosenblatt, 2007).

In HD patients, the most common psychiatric manifestation is the executive dysfunction syndrome, which causes apathy, irritability, perseverance, impulsiveness, and obsession. In addition, there are other non-specific psychiatric symptoms of HD. For example, HD leads to the development of delirium, which is a sudden change in mental status characterized by confusion and disorientation. The safest pharmacologic treatment for delirium is low-dose neuroleptics. HD also causes demoralization, and it's usually more intense at times of loss, such as when the patient is forced to stop working or give up driving (Rosenblatt, 2007).

Sexual problems are also present in HD patients. On one hand, they might present hypoactive sexual desire and inhibited orgasm, which have been reported in 63% and 56% of men, and 75% and 42% of women (Rosenblatt, 2007). On the other hand, HD patients might also present sexual aberrations like sexual assault, promiscuity, incest, indecent exposure, and voyeurism. Finally, HD patients frequently have sleep problems. Many have insomnia for a wide variety of reasons, which include depression, involuntary movements, and lack of daytime stimulation (Rosenblatt, 2007).

Current Treatment Approaches of Huntington's Disease

Even though there still isn't a definite cure for HD, there are treatments for the physiological effects that it causes. These mainly involve drugs that are consumed and technologies to target and edit the gene that causes the disease. It is important to note that these treatments can ameliorate the symptoms of HD, especially those related to neurodegeneration,

but they cannot eliminate the disease. In the following subsections, the available treatments currently will be mentioned, specifically those for excitotoxicity, caspases, HTT aggregates, mitochondrial dysfunction, transcriptional dysregulation, and the targeting of the mHTT gene in DNA. It's important to consider these available treatments to evaluate CRISPR-Cas9 as another potential treatment and possible cure later in the research project.

Drugs that Prevent Excitotoxicity and Target Caspase

Excitotoxicity refers to the pathological process where neurons are killed due to the overstimulation of neurotransmitters like glutamate. Specifically, the expansion of CAG leads to an excess of glutamate and protein aggregates, and this phenomenon causes the overstimulation of the neurotransmitter. Some drugs are known to inhibit glutamate activity and this way prevent excitotoxicity.

Memantine, which is a medication that works as an antagonist of extrasynaptic N-methyl-D-aspartate (NMDA) receptors, can decrease striatal cell death and prevent HD progression. NMDA receptors are associated with glutamate activity, so memantine blocks excessive activity of these receptors and glutamate stimulation in general. In a study conducted on rodents, researchers found that HD pathology can be reduced by consuming memantine on a low dose. However, the same study determined that memantine in a high dose can lead to cell death (Serdar et al., 2022).

There are inhibitors of vascular monoamine transporter type 2, such as tetrabenazine (TBZ) and deutetrabenazine. Vascular monoamine transporter type 2 (VMAT2) is a protein found in the membranes of synaptic vesicles that transports neurotransmitters from the cytoplasm of the neuron into synaptic vesicles. These proteins are related to an increased activity of neurotransmitters like glutamate and dopamine, meaning that drug inhibitors of VMAT2 are an

effective treatment for HD because they regulate excessive activity of neurotransmitters as well. In studies of mouse models, it was found that TBZ improved motor symptoms and reduced striatal neuronal loss (Serdar et al., 2022).

Excessive neurotransmitter activity usually leads to neuronal death, also known as apoptosis. Apoptosis is a form of programmed cell death in which cells undergo a regulated process of self-destruction, and it is a process supported by caspase proteins. Some drugs inhibit caspase activity to control the cell death that is caused by HD. One example of these drugs is minocycline, also known as tetracycline analog, which inhibits caspase 3 and caspase 1 activity. In a study conducted with humans, HD patients received 100 mg of Minocycline for 6 months, and motor and cognitive improvements were observed after this treatment (Serdar et al., 2022).

The availability of these drugs that target HD evidence that there are options to treat the physiological effects of the disorder, such as neurodegeneration, but there are no treatments that cure the disease completely. Since these drugs target the molecular mechanisms that are caused during the disease, they are successful in ameliorating the motor and cognitive symptoms. However, none of these treatments deal with the underlying cause of the problem, which is the DNA sequence in the HTT gene. This is the reason why they are not successful in curing the disease completely.

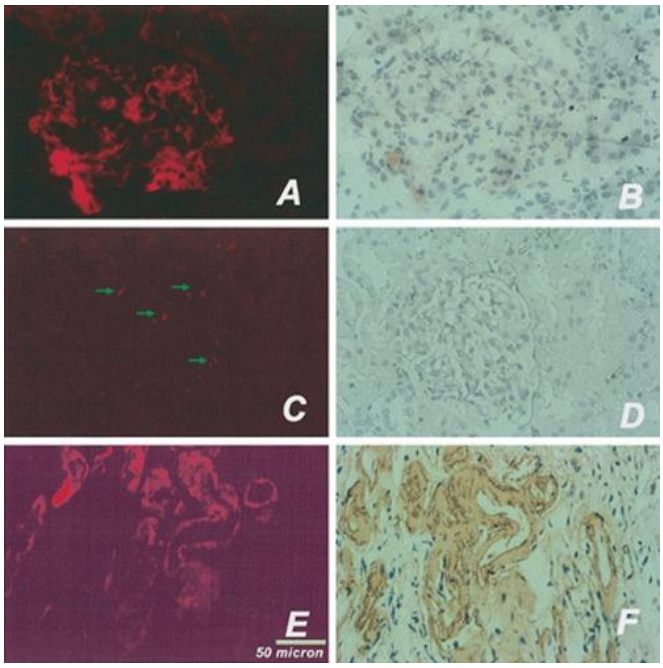
Targeting of HTT Aggregates

HTT aggregates represent the misfolded HTT proteins that are produced during HD. It is known that, when the dye called Congo Red is injected into mice with HD, it is effective in preserving normal protein synthesis and degradation, preventing the formation of aggregates. The Congo Red dye has a unique affinity for amyloids, which are a type of protein aggregates that are formed in HD. When the dye binds to amyloid fibrils, it helps identify the disease in the

patient. In addition, Congo Red helps the clearance of the polyQ expansion and prevents caspase activation and ATP exhaustion (both related to neuronal death) (Serdar et al., 2022). The following figure displays how Congo Red looks in the images of a study:

Figure 7

Congo Red Fluorescence



Note: Both sections (right and left) are stained with Congo Red, but they look differently because the left side is illuminated with ultraviolet (UV) light, while the right side is illuminated with bright light.

Source: (Gate, n.d.)**.**

This is a graphic representation of how Congo Dye is used to identify targets in body structures, and how high-quality images are helpful to make its use more effective.

The mammalian target of rapamycin (mTOR) is a protein kinase (phosphorylates other compounds) that plays an important role in cellular functions like transcription and autophagy (recycling of the cell's components). In the context of HD, mTOR can be overactive and reduce

the cell's ability to clear toxic aggregates. It is known that mTOR deregulation is associated with HD: "mTOR disorder, which is known to be associated with mHTT, is an important factor in explaining brain atrophy in HD." Scientists have evaluated drugs to treat this issue and they have found Rapamycin successful in HD mouse models because it develops motor performance and decreases striatal neuropathology (Serdar et al., 2022).

The availability of these treatments that include drugs and fluorescent staining again proves that there are options to treat the molecular effects of HD, in this case, the production of protein aggregates specifically. Since protein aggregates are so toxic and lead to neurodegeneration, using treatments that regulate them is highly effective in controlling the symptoms of HD. However, as mentioned before, this type of treatment can only ameliorate the symptoms of the disorder, not cure the disease completely.

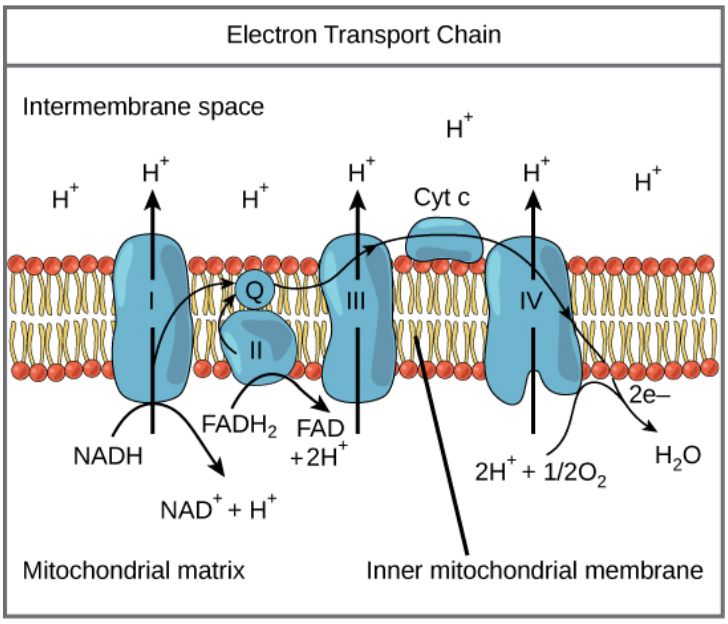
Targeting of Mitochondrial Dysfunction

Before the current treatments for mitochondrial dysfunction in HD are analyzed, it's important to explain and understand the mechanisms or processes involved in mitochondrial failure. Mitochondria are the primary source of energy (ATP) in cells, and this is generated through the process of oxidative phosphorylation.

During HD, mHTT disrupts the function of Complex II and Complex IV of the electron transport chain (step of oxidative phosphorylation during energy production), directly reducing ATP production in neurons and leading these cells to have an energy deficit. Neurons, particularly those located in the basal ganglia, require high amounts of ATP. Since HD mainly affects the basal ganglia, these neurons are severely affected and their lack of energy contributes to the motor and cognitive symptoms of the disease.

Figure 8

Oxidative Phosphorylation



Note: The image displays the complexes II and IV of the electron transport chain, which are affected during HD. They are essential for the production of ATP, as they have the key roles of decomposing $FADH_2$ and transporting electrons through the membrane.

Source: (Jung, 2020)**.**

Dysfunctional mitochondria during HD produce excess reactive oxygen species (ROS) as a result of the failure in the electron transport chain of cellular respiration. ROS are chemically reactive molecules containing oxygen that can cause cellular damage when in excess. For this reason, elevated levels of ROS cause oxidative stress by damaging cellular components like DNA, proteins, and lipids, accelerating neuronal death.

Additionally, the mitochondrial function of releasing calcium ions to maintain the membrane potential is disrupted by mHTT. The mitochondrial membrane potential is the electrical potential difference across the inner mitochondrial membrane, and it's generally negative on the inside relative to the cytoplasm. To maintain this negativity inside the

mitochondria, calcium ions, which have a positive charge, are released. mHTT sensitizes mitochondria to calcium overload, and this leads to a process called mitochondrial permeability transition, where the mitochondrial membrane potential is disrupted and apoptosis is triggered as a consequence (M.R., 2000).

Studies have shown that creatinine, which has antioxidant properties, delays the development of HD and improves muscle movement capacity. However, other studies contradict this, since they show that creatinine isn't useful in improving cognitive decline and neuromuscular functions in stages I-III of HD. Other treatments like coenzyme Q cofactor and Ethyl-Eicosapentaenoic Acid (EPA) have shown effectiveness in regulating mitochondrial dysfunction in some studies, but other studies didn't determine this efficiency. It has been shown that cystamine blockers increase the survival rate of neurons and Meclizine inhibits oxidation and apoptosis, meaning that they're useful in treating HD (Serdar et al., 2022).

Targeting of Transcriptional Dysregulation

Transcriptional dysregulation refers to the disruption of normal gene expression patterns within a cell. The wild-type HTT protein usually interacts with transcription factors and other transcriptional regulatory proteins. The mutated HTT protein disrupts this process, and, since the genes that are regulated usually code for proteins that are crucial for cell survival, this failure leads to the death of neurons. Now that the concept of transcriptional dysregulation in HD has been explained, some potential drugs for this effect will be considered.

In a study performed with N171-82Q symptomatic mice, sodium phenylbutyrate led to a decrease in brain atrophy and regulation of caspases, reducing apoptosis (Serdar et al., 2022). In HD, there are low levels of histone acetylation, which makes chromatin more condensed and limits access to genes that are essential for neuronal function and survival. Therefore, drugs that

inhibit deacetylases or promote acetylation are useful to treat transcriptional dysregulation. For example, other studies with mouse models have determined that DACi4b, a histone deacetylase (HDAC) inhibitor, reduces neurodegeneration and regulates mRNA expression (Serdar et al., 2022).

Targeting of DNA

Approaches that target DNA include Zinc Finger Nucleases (ZFNs), TALENs, and CRISPR-Cas9. Each of these approaches is different regarding DNA binding and modes of action. In the following, the different modes of action of these three genome editing methods will be explained.

ZFNs are artificial enzymes that are used in molecular biology to edit DNA with high precision. Each zinc finger can recognize and bind to a sequence of three base pairs, so scientists link many zinc fingers to recognize a long DNA sequence. An endonuclease called Fok-1, which is a DNA-cutting enzyme, is attached to the zinc fingers, and it waits until two ZFNs bind to opposite strands of DNA to form a dimer. This dimer then creates a double-strand break at the target site and removes the non-wanted sequence. In the case of HD, ZFNs are designed to be selectively linked to CAG repeats, and, this way, they end up binding to the mHTT gene. Even though ZFNs can reduce mHTT levels, they cannot change DNA, which is a limit for the technique (Serdar et al., 2022).

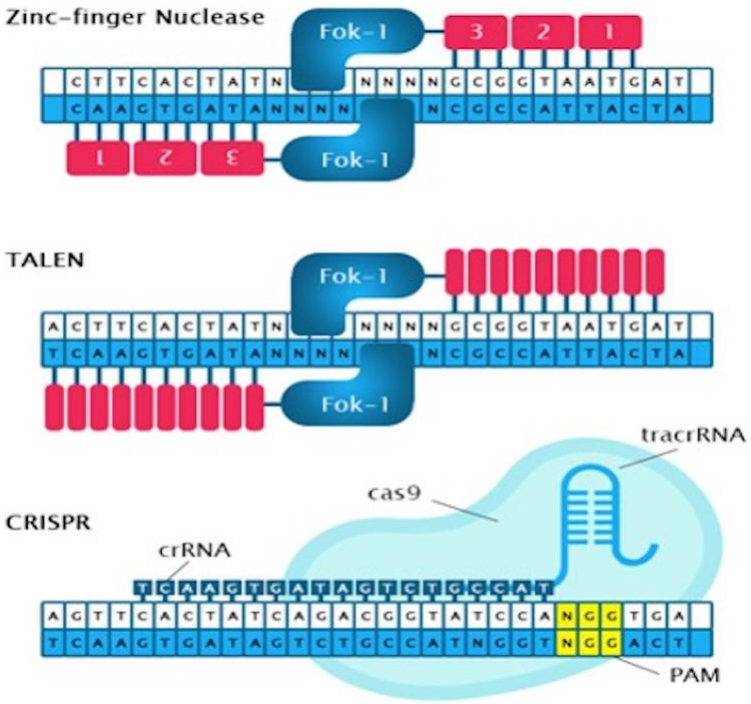
Alternatively, Transcription Activator-Like Effector Nucleases (TALENs) are a type of engineered protein that recognizes and cuts specific DNA sequences within the genome. TALENs use proteins known as transcription activator-like effectors (TALEs), and each TALE is designed to bind to a specific nucleotide in DNA. When multiple TALE repeats are combined, TALENs can target a precise sequence, and then Fok-1 (the same endonuclease that's used in

ZFNs) comes in, forms a dimer with two TALENs, and cuts the target sequence. In the case of HD patients, TALENs cut the mutant HTT gene that is causing the disorder. It is known that the TALEN-based approach is more efficient than ZFNs, but TALENs need a specific nucleotide and this may limit targets at the end of the DNA sequence (Serdar et al., 2022).

CRISPR-Cas9 is a genome-editing system that can target specifically any region of DNA, which gives it an advantage over TALENs and ZFNs. The technology starts with the design of a single-guide RNA (sgRNA) that matches the target DNA sequence, and this sgRNA binds to the Cas9 enzyme, which acts as molecular scissors to cut DNA. The guide RNA is composed of two parts: crRNA (CRISPR RNA), which contains the sequence that is complementary to the target DNA, and tracrRNA (trans-activating CRISPR-RNA), which helps in the binding of crRNA to Cas9. When the sgRNA finds its complementary DNA sequence, Cas9 cuts both strands of DNA at that specific location; this is known as a double-strand break. After Cas9 cuts the DNA, the cell uses two repair mechanisms, which are non-homologous end joining (NHEJ) and homology-directed repair (HDR). These mechanisms will be explained in more detail later in the theoretical framework. It's important to note that the complex recognizes the target gene where there is the PAM sequence, which is the trinucleotide NGG (any nucleotide, guanine, guanine). In the case of HD patients, CRISPR-Cas9 allows the deletion of the mHTT gene and the correction of DNA afterward. The following image displays the differences between ZFNs, TALENs, and CRISPR-Cas9, highlighting the enzymes and molecular mechanisms involved in the tools:

Figure 9

ZFN, TALEN, and CRISPR Genome Editing Approaches



Note: The ZFN technique shows the Fok1 endonuclease and the zinc fingers that target three nucleotides each. The TALEN technology displays the Fok1 endonuclease and TALEs that target one nucleotide each. Finally, the CRISPR method shows the PAM sequence, sgRNA (composed of tracrRNA and crRNA), and Cas9.

Source: (Serdar et al., 2022)**.**

When compared to ZFNs and TALENs, CRISPR-Cas9 provides evident advantages as a treatment for HD, since it can target any region of DNA and it's more simple to design. In the following section of the theoretical framework, CRISPR-Cas9 technology will be explained in detail.

Mechanisms and General Applications of CRISPR-Cas9

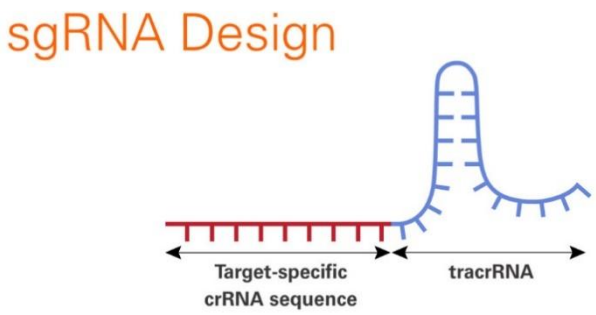
CRISPR-Cas9 is a commonly used genome-editing tool in biomedical research. It allows doctors to correct DNA mutations to treat or cure diseases. Besides the treatment of genetic disorders, CRISPR-Cas9 is also used in immunology-focused therapies to support anti-tumor immunotherapy and AIDS treatment (Serdar et al., 2022). This section will talk about the molecular mechanisms underlying this technology, the repair pathways of DNA, and the potential combination of CRISPR with Induced Pluripotent Stem Cells (iPSCs).

CRISPR-Cas9 Technology Mechanisms

The CRISPR-Cas9 system has two basic components: sgRNA (single-guide RNA) and Cas9 nuclease. The Cas9 nuclease depends on the sgRNA, which is made specific to the target DNA sequence. Cas9 induces breaks or notches in any region of DNA *in vivo*. On the other hand, sgRNA determines the specificity of target DNA by linking to complementary DNA sequences through nucleotide base pairing (Serdar et al., 2022). The following figure shows the composition of sgRNA:

Figure 10

SgRNA's Composition-crRNA and tracrRNA



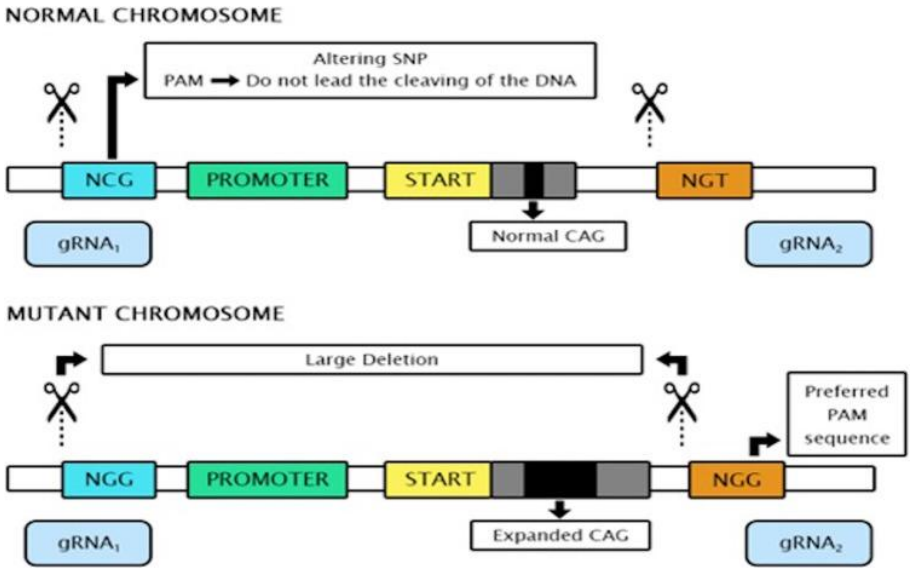
Source: (Takara, n.d.)**.**

Cas9 connects to sgRNA and it causes DSB (double-strand breaks) in the DNA backbone (pentosugar and phosphate group). DSBs are breaks in both strands of the DNA double helix.

The Cas9-sgRNA complex finds the target DNA sequence by recognizing a specific region known as the PAM sequence (located at the beginning of the gene). PAM usually contains between 2 and 5 nucleotides, and the sequences it might have are NGG (any nucleotide, guanine, guanine) or NAG (any nucleotide, adenine, guanine) (Serdar et al., 2022). The required PAM sequence varies with the Cas protein that's being used; in the case of Cas9, it requires the PAM sequence NGG or NAG. The following figure shows the difference between the structure of a normal chromosome and a mutant chromosome, and how this affects the recognition of the PAM sequence and the cleavage of the expanded CAG repeat sequence:

Figure 11

CRISPR-Cas9 Approach to Reduction and Permanent Inactivation of HD



Note: The normal chromosome displays a normal CAG length and a non-preferred PAM sequence, while the mutant chromosome shows an expanded CAG length, a preferred PAM sequence, and a large deletion.

Source: (Serdar et al., 2022)**.**

Single-guide RNA and Cas9 are introduced to the cells through vectors using recombinant DNA technology. Vectors are delivery systems that can include several types, such as viral vectors and plasmid vectors. In the case of viral vectors, the components for CRISPR are inserted into a virus that has been engineered to be safe and non-replicating. When the virus infects the cell, it releases the CRISPR components and integrates them into the DNA of the host. On the other hand, plasmids are circular DNA molecules. When used as plasmid vectors, they are inserted into the person's DNA and transcribed so that they produce the Cas protein and sgRNA.

These vectors are inserted into the patient's DNA using recombinant DNA technology, which is a set of techniques used to manipulate DNA from different sources. It allows scientists to modify the genetic makeup of an organism by introducing new genes, which might come from bacteria or other organisms. Recombinant DNA technology in CRISPR-Cas9 technology will be explained in the following.

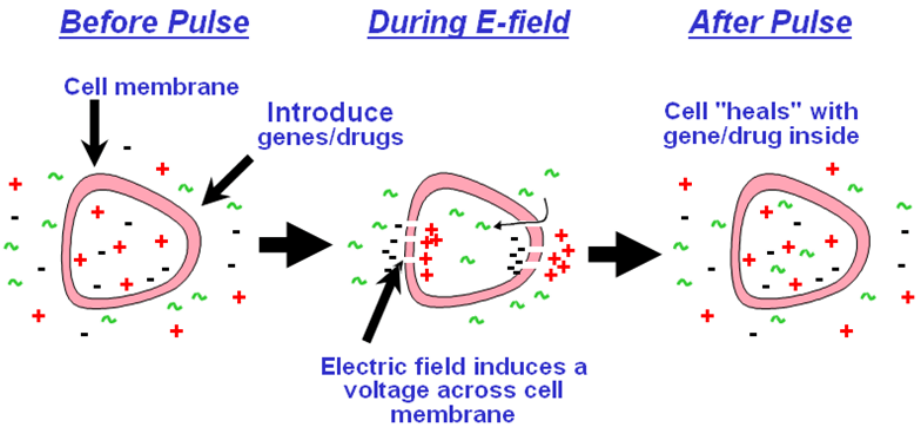
For recombinant DNA technology using CRISPR, the first step is to design the necessary components. The sgRNA must be designed, which, as mentioned before, must contain two parts: crRNA, which matches the target DNA sequence, and tracrRNA, which helps the system function. The Cas9 gene is obtained from the bacterium *Streptococcus pyogenes* or other organisms with similar systems, and scientists then clone this gene into a plasmid or another vector (Jinek, et al., 2012).

After this, the sgRNA is also inserted into the plasmid or the vector; sgRNA and Cas9 are sometimes inserted into the same vector, but other times they're in separate vectors. If the vector is a genetically modified virus or a plasmid, its genome must be cut by enzymes and the desired gene fragments (sgRNA and Cas9) must be inserted. The vector is then sealed with the help of DNA ligase. The vectors need to contain promoter sequences since these allow for the expression of the Cas9 gene and sgRNA in the target cells.

Subsequently, the vectors are introduced into host cells through methods like electroporation, lipofection, microinjection, or gene gun techniques. Electroporation is based on applying a short electrical pulse that opens pores in the cell membrane for molecules to enter the cell. The following figure displays the procedure of electroporation:

Figure 12

Electroporation

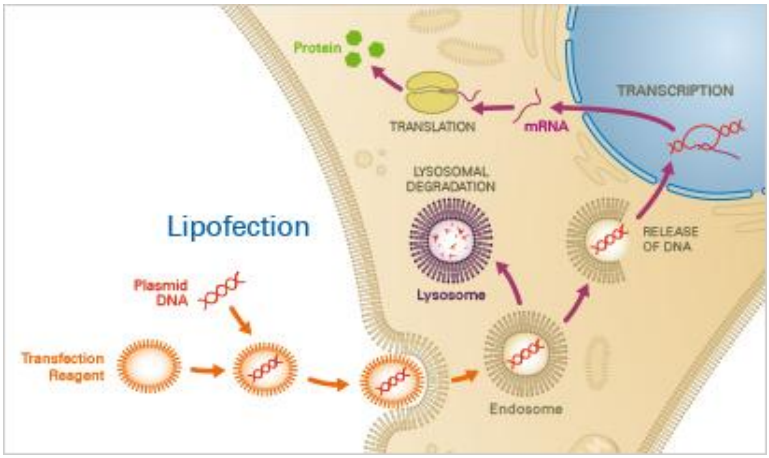


Note: (SMI, n.d.)**.**

Conversely, lipofection uses lipid-based carriers (liposomes) that fuse with the cell membrane, allowing the material to come into the cell. The following image shows the step-by-step process of lipofection. It's important to note that the transfection reagent is the same as the liposome or lipid-based carrier mentioned previously.

Figure 13

Lipofection



Source: (Ibidi, n.d.).

Other methods include microinjections and gene guns. Microinjections directly inject substances into individual cells by using a fine needle or micropipette. Finally, gene guns are devices that deliver material into cells by shooting tiny particles (usually gold or tungsten) coated with DNA.

Once inside the cells, the genes that code for Cas9 and sgRNA are transcribed and translated. Then, the Cas9 protein will be activated and guided by the sgRNA to the target DNA sequence. The crRNA does a base pairing with the target DNA and Cas9 creates a DSB at the target site, causing the target sequence to be cut (Serdar et al., 2022). After this, the cell's natural repair mechanisms take over, which will be explained in the following subsection.

Even though CRISPR-Cas9 has a very specific procedure, the components involved are easy to obtain and the steps can be followed in the laboratory. Based on the previous explanation, it can be affirmed that Cas9 is an enzyme that can be manipulated easily and that can cut any

region of DNA. Also, scientists can design sgRNA according to the gene that must be edited, making it a flexible method that accommodates to the interests of the researchers. CRISPR-Cas9, if it continues to develop, can be the breakthrough in biomedical technology, making it a very useful tool for diagnostics, research, and therapeutics.

DNA Repair Pathways Following CRISPR-Cas9

The DSBs are repaired through two possible natural mechanisms: non-homologous end joining (NHEJ) or homologous-directed repair (HDR). In NHEJ, the Ku heterodimer, which is composed of the proteins Ku70 and Ku80, binds rapidly to the broken DNA ends to protect them and recruit additional repair proteins, such as XRCC4:

> Ku physically interacts with the XRCC4-DNA Ligase IV complex and recruits it to the DNA ends *in vitro* and *in vivo.* XRCC4 may be a second NHEJ scaffold which is responsible for the recruitment of a number of NHEJ factors to the DSB factors to the DSB ends (CD, n.d.).

Other factors, like DNA-PKcs, also stabilize the NHEJ complex.

If necessary, the next step is to process the DNA ends to create ligatable ends. Recruited most likely by a Ku-XRCC4 scaffold, DNA end processing enzymes can resect DNA ends, fill in gaps, make the ends ligatable, and remove blocking end groups. Some of these processing enzymes involve Ku and the AP lyase. Other proteins involved in the resecting of DNA are Artemis, WRN, and APLF (CD, n.d.).

Finally, the broken ends are connected again by DNA ligase IV. XRCC4 stabilizes and stimulates the activity of ligase IV by promoting its adenylation (attachment of an adenosine monophosphate, or AMP). Even though there is little information available about the

mechanisms that allow the dissolution of the NHEJ complex (Ku-XRCC4 molecule), human-cell research showed that E3 ubiquitin ligase RNF8 might mediate this process. After the broken ends are connected, DNA has been repaired and has gone back to its normal state (CD, n.d.).

NHEJ is more prone to error because it has a high probability of insertion or deletion (INDEL) mutations (Serdar et al., 2022). This happens because, unlike HDR, NHEJ doesn't rely on a homologous template to repair the break. Now, HDR will be explained and then compared to NHEJ.

HDR is initiated by the resection of the 5' DNA end of the break by nucleases, which then creates a single-stranded DNA (ssDNA) 3' overhang. SsDNA 3' overhang is a section of DNA where one strand of DNA extends beyond the other, leaving a short, single-stranded tail with a free 3' end. SsDNA overhangs are coated with the RAD51 protein, which helps search for a homologous sequence; once this homologous sequence is found, ssDNA overhang invades it. This invasion forms a D-loop (displacement loop), a DNA structure where the invading strand pairs with its complementary strand (Gearing et al., 2015).

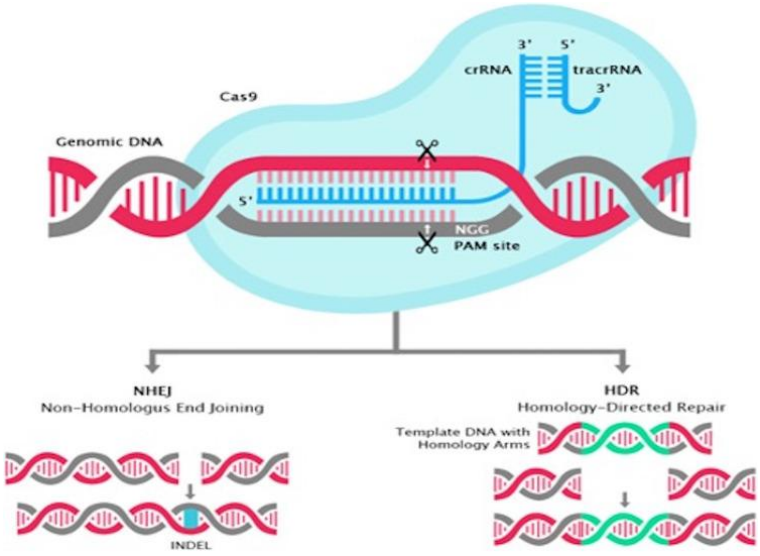
After, DNA polymerases extend the 3' end of the invading strand using the homologous template as a guide, and, as the new DNA is synthesized, it replaces the missing section at the break site. This new DNA sequence is re-ligated with the original broken DNA ends, and DNA ligases help to seal any remaining gaps, leaving DNA completely repaired (Gearing et al., 2015).

HDR provides greater precision and accuracy than NHEJ because it uses a homologous DNA sequence as a template to restore the original DNA sequence. It also has greater control in gene editing because it can insert specific sequences, correct mutations, or knock in a gene. All of this minimizes the risk of off-target mutations at the site, which are more prone to occur as

INDEL mutations in NHEJ. However, HDR does have disadvantages when compared to NHEJ. It is less efficient because it can only occur in the S and G2 phases of the cell cycle, while NHEJ can occur throughout the whole cell cycle, making it faster and more widely applicable. Additionally, it is a more complex and slower repair process because it requires additional steps, such as homology search, alignment, and template incorporation. The following figure displays the differences mentioned above between NHEJ and HDR:

Figure 14

DNA Repair Mechanisms in Gene Editing Systems

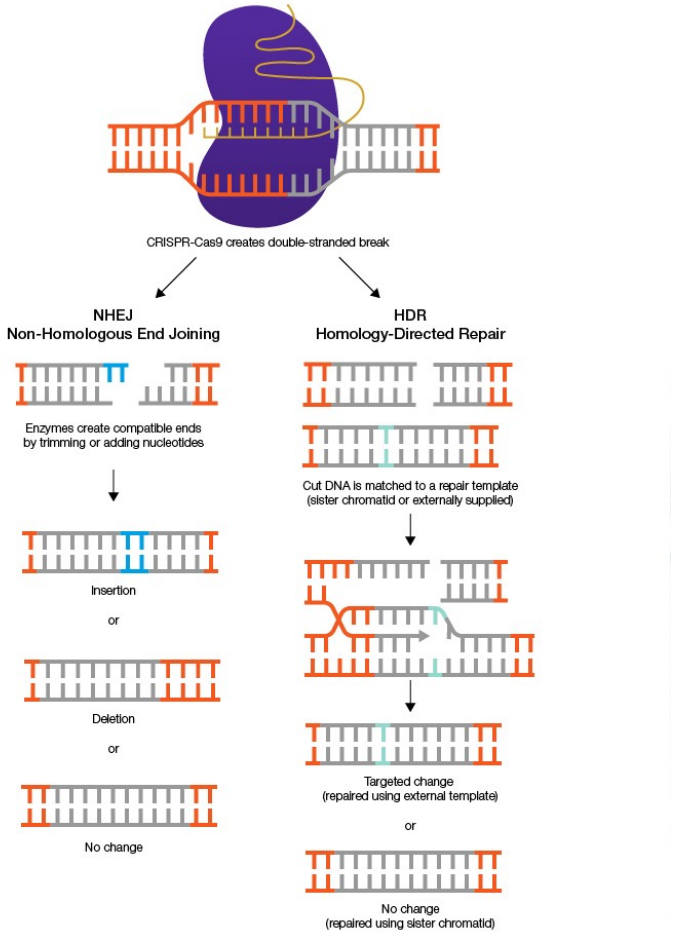


Source: (Serdar et al., 2022)**.**

The following figure also shows the difference between these two methods, while also presenting the procedure that they follow:

Figure 15

Homology Directed Repair (HDR) vs Non-Homologous End Joining (NHEJ)



Source: (Alexander, 2020).

Now that the whole procedure of CRISPR-Cas9 and its repair mechanisms were explained, the research project will consider the current integration of CRISPR with iPSCs, which provides great potential for the medical field in the future.

Integration of CRISPR-Cas9 with Regenerative Medicine – iPSCs

Regenerative medicine is a field of medical science that focuses on repairing damaged cells, tissues, and organs to restore normal function. This is approached using stem cells, which are undifferentiated cells with the capacity to give rise to more cells of the same type and differentiate into specified cell types, and this gives them the capacity to regenerate. In stem-cell-based therapeutic strategies, stem cells are cultured and delivered to the target specific organ by direct injection.

Induced Pluripotent Stem Cells (iPSCs) have become one of the stem cells that form the basis of regenerative medicine (Serdar et al., 2022). They are used in various disorders, such as organ failures, spinal cord injuries, neurological damage, and skin disorders. IPSCs are created by reprogramming adult cells, such as skin or blood cells, back into a pluripotent state, meaning that they can differentiate into almost any cell type in the body. The only difference between iPSCs and normal stem cells is that they're derived from humans rather than embryos.

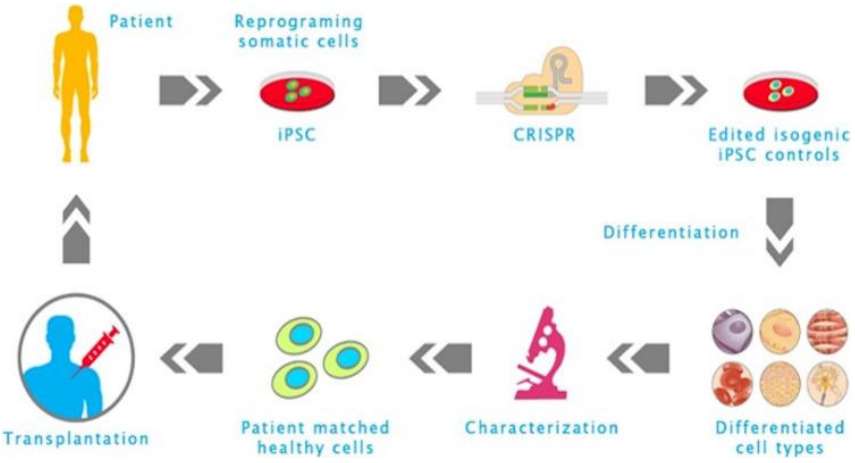
CRISPR-Cas9 technology results in iPSC applications, and they can be used specifically for HD since they play an important role in neurodegenerative diseases. CRISPR-Cas9 can edit the genome of cultured stem cells of the patient, and these can then be used as regenerative drugs in therapeutic applications. IPSCs have been used *in vitro* for the modeling of neurodegenerative disorders, and CRISPR-Cas9 has been used *in vivo* in the modeling of human diseases and the production of genome-regulated animals carrying the genetic mutations that cause the disorder. CRISPR-Cas9 intends to correct the mutations that cause diseases *in vivo*, and iPSCs are the vehicle that allows these corrections to be done.

The technology of iPSCs starts by obtaining the patient's somatic cells, which will then be reprogrammed to iPSCs so that disease-related cells can be produced *in vitro*. These cells are flexible and can have diverse uses: "Human iPSCs can differentiate into any cell type in the human body…iPSCs can be used in human disease models such as developmental and adult diseases." (Serdar et al., 2022). IPSCs can be used in several forms: two-dimensional (2D) cell cultures, three-dimensional (3D) organoids, phenotype-based drug screening, and to determine the phenotypes of neurodegenerative diseases. Taking into account the efficiency of CRISPR-Cas9 and the utility of iPSCs, combining these two methods to treat diseases is a viable option.

The steps that must be followed to combine CRISPR-Cas9 and iPSCs are as follows. First, as mentioned previously, somatic cells from the patient must be obtained, and they will be reprogrammed to iPSCs. Then, the iPSCs will be genetically modified using CRISPR-Cas9, this to correct the mutations that are causing the disease. After, the modified iPSCs are differentiated into the necessary type of cell, and they go through characterization, where it's confirmed that they have followed the desired lineage. Finally, the patient-matched healthy cells are transplanted into the patients through methods like direct injection (Serdar et al., 2022). The following image shows this procedure graphically:

Figure 16

CRISPR Gene Editing with Human iPSCs



Source: (Serdar et al., 2022)**.**

CRISPR-Cas9 has a lot of potential to treat human diseases, and even more if it's combined with the iPSC technology, making it more effective and applicable to diverse cases. Now that the mechanisms and procedures of this gene-editing tool have been explained, its practical applications to cell and animal models with Huntington's Disease will be evaluated. While CRISPR-Cas9 seems useful from the theoretical perspective, it's important to also take into account the practical applications that have used this method up to today and the investigations that are missing to establish it as a therapy or treatment for HD in clinical centers.

Huntington's Disease Treatment Approach in Cell and Animal Models with CRISPR-Cas9

There have been several investigations and research projects to study CRISPR-Cas9 as a treatment for HD. These have been both *in vivo* and *in vitro* applications of the method. *In vivo* studies have been done within living organisms, mainly in mice, while *in vitro* studies have taken place in laboratory cultures of cells. In the following subsections, some of these investigations

that have been developed up to today will be presented and analyzed, to determine the effectiveness of CRISPR-Cas9 as a treatment for HD.

HTT Gene Editing Using CRISPR-Cas9 in iPSCs and MSN Formation

It is known that the most affected cells in HD are medium spiny neurons (MSNs), since they degenerate as the disease develops. MSNs can be obtained through iPSCs: somatic cells from HD patients are obtained, they are reprogrammed back into their pluripotent state, and they are again reprogrammed to become MSNs. In addition, the phenotypes of neurons of HD patients can be studied through iPSCs. Scientists derive MSNs from HD patients' iPSCs to model the disease *in vitro*, allowing them to study HD-related cellular phenotypes, such as neuron degeneration and the formation of protein aggregates.

Although the modeling of HD *in vitro* has improved, it remains rare to observe the formation of mHTT protein aggregates in HD-iPSC-derived neurons. To improve the accuracy of disease modeling using iPSCs, scientists have created isogenic pairs of the cells, including the original HD iPSCs and genetically corrected counterparts (using CRISPR-Cas9) to repair the CAG repeat region. These iPSC-derived MSNs that have been corrected with CRISPR-Cas9 can be used to study the disease since they show recovery in HD phenotypes, such as the correct functioning of the mitochondria (Serdar et al., 2022).

The article by Serdar, S., Alkanli, N., Ay, A., Albeniz, I. (2022) CRISPR/Cas9 Mediated Therapeutic Approach in Huntington's Disease from *Molecular Neurobiology* reported that several studies have shown that HD-iPSCs can be genetically modified using CRISPR-Cas9. Some studies used HDR to repair DNA after CRISPR-Cas9 corrected the mHTT gene, and, after

this, antibiotic selection was used to ensure only successfully-edited cells were retained for further studies.

Other investigations have used the Cas9 D10 nickase variant, which creates single-strand breaks instead of double-strand breaks. This variant has been used because the DSBs produced by the wild-type Cas9 protein can be error-prone and induce unintended genetic changes at off-target sites, while single-strand breaks enhance specificity and reduce the risk of off-target mutations. The Cas9 D10 nickase variants have been paired with sgRNAs, and it has edited the DNA of HD patients with more precision. This was important because it provided insights into the possible use of CRISPR-Cas9 as a treatment for HD but with the D10 nickase variant to prevent dangerous mutations.

In another approach, fibroblasts (connective tissue cells) were obtained from HD patients and they were modified using CRISPR-Cas9 to excise the mutant HTT gene containing the CAG expansion. Researchers inactivated the mutant allele and were successful in reducing the harmful effects of the abnormal HTT protein, such as mitochondrial dysfunction and apoptotic activity (Serdar et al., 2022).

These studies are examples of the success of CRISPR-Cas9 to modify HD-affected cells, whether it's through the use of iPSCs or not. They also provide useful information about the benefits of using the Cas9 variant D10 nickase, which might be less risky than the wild-type Cas9. These discoveries are useful in the process of developing CRISPR-Cas9 as a treatment for HD in clinical centers. Now, another study of CRISPR-Cas9 for HD treatments will be analyzed, this time using mouse models.

Disruption of uORF in MSCs from YAC128 HD Mice Using CRISPR-Cas9

The study that will be described in the following subsection was reported in Kolli, N., Lu, M., Maiti, P., Rossignol, J., Dunbar, G. (2017) CRISPR-Cas9 Mediated Gene-Silencing of the Mutant Huntingtin Gene in an In Vitro Model of Huntington's Disease from the journal *Molecular Sciences*. The investigation was done in mesenchymal stem cells (MSCs) extracted from the bone marrow of YAC128 mice that carried mHTT in their genomes. MSCs are stromal cells, which make up connective tissue, that can self-renew and differentiate into various lineages. These were extracted from the bone marrow, the soft, spongy tissue that is found in the centers of bones. The organisms that were used for this experiment were YAC128 mice, which are genetically engineered mouse models used to study HD. These mice carry a large fragment of the human HTT gene that includes the expanded CAG repeat, as in HD patients (Kolli et al., 2017).

For the experiment, researchers constructed two CRISPR-Cas9 plasmids; one of the CRISPR-Cas9 plasmids was in charge of cutting DNA at the untranslated region upstream to the open reading frame (uORF), and the other had the role of cutting DNA at the exon 1-intron boundary (location of mHTT) (Kolli et al., 2017). The upstream open reading frame (uORF) is a short sequence in the 5' untranslated region (5' UTR) of an mRNA that will be translated; it is located upstream of the main coding sequence (main uORF) of a gene. During the experiment, these CRISPR-Cas9-containing plasmids were injected into the MSCs of the YAC128 mice mentioned previously, this to observe if CRISPR-Cas9 can be effective in editing the mutant HTT gene.

It had been previously reported that 5' Untranslated Region (UTR) of the HTT gene is crucial in regulating the synthesis of the HTT protein. The presence of the upstream uORF can

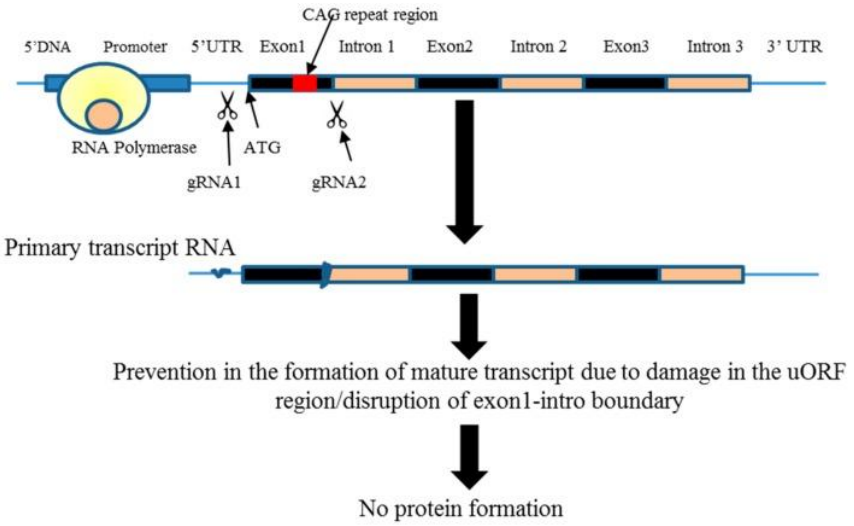
affect the translation of the downstream uORF (the HTT gene in this case). Therefore, the researchers in this experiment hypothesized that the CRISPR-Cas9 plasmids could disrupt the uORF present in the 5' UTR of the mHTT gene and that this would reduce the translation of the HTT protein.

As the researchers injected the designed Lenti-CRISPR-Cas9 (system that combines CRISPR-Cas9 machinery and lentiviral vectors to inject them) in the MSCs derived from YAC128 mouse models, they observed that uORF expression was disrupted (Kolli et al., 2017). This process activated stop codons that terminated the translation of the mutant gene. Stop codons are specific sequences of nucleotides that signal the end of translation by marking where the ribosome should stop synthesizing a protein. Three stop codons are UAA, UAG, and UGA.

Scientists designed another Lenti-CRISPR-Cas9 that targeted the exon1-intron boundary, which is the location of the mHTT gene. CRISPR-Cas9 was successful in cutting the mutant gene, preventing its presence in the mature mRNA transcript, and also preventing its translation into the mHTT protein, as displayed in the following figure:

Figure 17

CRISPR-Cas9 Injection in MSCs of YAC128 Mouse Model



Note: The figure shows gRNA1, which disrupts uORF, and gRNA2, which disrupts the mHTT gene.

Source: (Kolli et al., 2017)**.**

This study proved that CRISPR-Cas9 can be effective in targeting the gene that causes HD, even if it's by cutting the region upstream that regulates its expression (uORF) or the mHTT gene itself. In the investigation, the Lenti-CRISPR-Cas9-mediated silencing of mHTT reduced dramatically the production of mHTT protein in the YAC128 mice MSCs (Kolli et al., 2017). These mice are genetically modified to contain the human HTT gene that causes HD, meaning that this experiment was a significant advancement about the possible effectiveness of CRISPR in humans. Since CRISPR could cut this human gene in mice, it can be inferred that it's possible to do the same in human HD patients that contain the same gene. Another significant discovery of the study was that CRISPR-Cas9 treatment can be more effective if both the coding region

and the upstream region that regulates it (uORF) are disrupted. This strategy can be applied in the future to HD patients in clinical centers for greater effectiveness of the treatment.

Now that an *in vitro* study in cultured mesenchymal stem cells has been studied, it's important to also consider CRISPR-Cas9 investigations *in vivo*, this to observe the physiological effects that the treatment has on the whole organism. For this purpose, another experiment involving the use of mouse models and the injection of CRISPR-Cas9 machinery into their brains will be studied. The following investigation also includes a CRISPR-Cas9 application *in vitro* with human cells.

CRISPR-Cas9-Mediated Genome Editing Increases Lifespan and Improves Motor Deficits in a Huntington's Disease Mouse Model

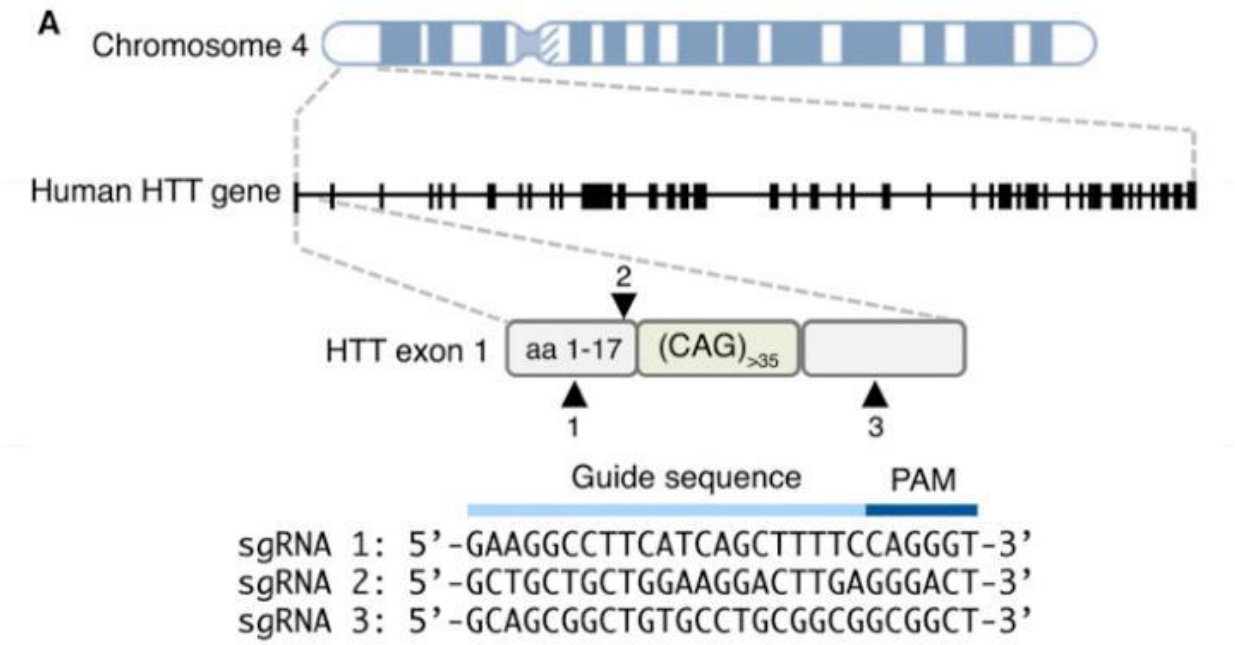
An application of CRISPR-Cas9 *in vivo* in mouse models is explained in the article by Ekman, F. K., Ojala, D. S., Adil, M. M., Lopez, P. A., Schaffer, D. V., Gaj, T. (2019) called CRISPR-Cas9-Mediated Genome Editing Increases Lifespan and Improves Motor Deficits in a Huntington's Disease Mouse Model from the *American Society of Gene & Cell Therapy (ASGCT)*. This scientific article explains how CRISPR-Cas9 was used to disrupt the expression of mHTT in human cells and the R6/2 mouse model after Cas9 was delivered *in vivo* to the striatum of the mouse.

Researchers hypothesized that CRISPR could disrupt the expression of mHTT if it was delivered *in vivo* using an adeno-associated virus (AAV) vector, which is an engineered gene delivery vehicle that can intervene in certain structures of the brain, including the striatum, which is affected by HD. Researchers first used the Cas9 nuclease from *Staphylococcus aureus* (bacteria), which is known as SaCas9 and also designed sgRNAs to target exon 1 from the

mHTT gene (Ekman et al., 2019). They did this with a human cell line before working with mice. The following figure displays the sites of the genome where sgRNA and Cas9 acted during the experiment in human cells:

Figure 18

Disruption of the Mutant HTT Gene in Reporter Cells Using CRISPR-Cas9 Nucleases (A)



Note: This is a schematic representation of the location of the HTT gene in chromosome 4 and the sites where sgRNA and Cas9 intervened during the experiment. The arrowheads show the approximate binding site of sgRNA. PAM stands for Protospacer-Adjacent Motif and "aa" stands for amino acids.

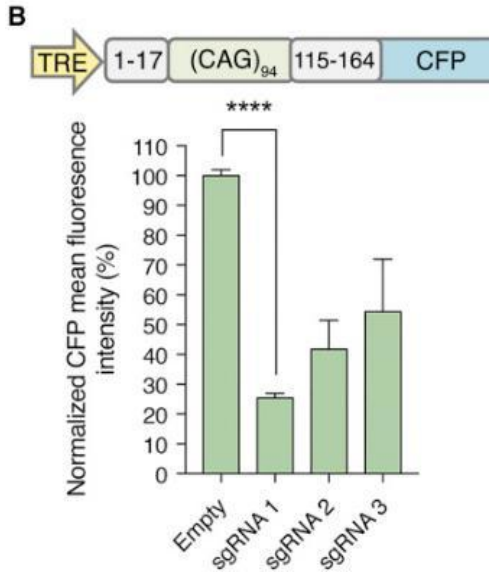
Source: (Ekman et al., 2019).

Since identifying the CAG trinucleotide repeat and the full-length HTT protein could be confusing, researchers linked the expression of mHTT to cyan fluorescent protein (CFP), which gives fluorescence to this region of the genome and enables the visualization of the gene through fluorescence level measurements (Ekman et al., 2019).

The sgRNAs were introduced into HEK293T cells (a human cell line) and successfully reduced the CFP fluorescence. “Following the transfection into HEK293T cells, we observed that each sgRNA decreased CFP fluorescence intensity by at least 50%...” (Ekman et al., 2019). These results proved that CRISPR-Cas9 was effective at reducing the expression of the mHTT gene and its transcription and translation levels. The following graph shows the results of CFP intensity after the application of CRISPR-Cas9, where each type of sgRNA caused a different percentage, but all of them reduced the previous mHTT expression:

Figure 19

Disruption of the Mutant HTT Gene in Reporter Cells Using CRISPR-Cas9 Nucleases (B)



Note: The graph evidences the percentage of CFP-positive HEK293T cells 72 hours after transfection by using SaCas9 and different types of HTTP-targeting sgRNAs or non-targeted sgRNA (empty).

Source: (Ekman et al., 2019).

These results indicate that SaCas9 can target the human HTT gene and reduce mHTT expression, providing evidence of the effectiveness of CRISPR therapy.

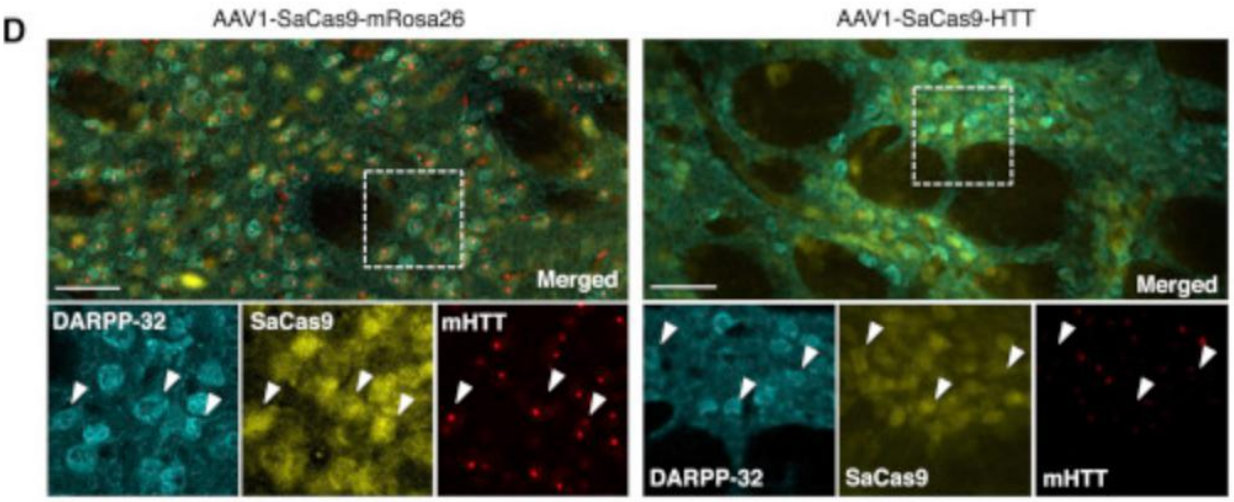
Subsequently, researchers evaluated the capacity of sgRNA to reduce mHTT expression in an R6/2 mouse model of HD, which contains the 5’ end of the human HTT gene. R6/2 mice develop a progressive neurodegenerative phenotype that is similar to HD in humans: “R6/2 mice develop HTT protein inclusions in the striatum and, eventually, the cortex and exhibit a

progressive neurodegenerative phenotype that mimics many features of HD in humans, including weight loss, tremors, epileptic seizures, movement abnormalities, and premature death." (Ekman et al., 2019). To prove this, researchers injected the striatum of these mice with the AAV1 vector with SaCas9 and two types of sgRNAs: AAV1-SaCas9-HTT, which targets the mutant HTT gene, and AAV1-SaCas9-mRosa26, which served as a control for the experiment (Ekman et al., 2019).

Four weeks after the delivery of AAV vectors, researchers used immunohistochemistry (IHC), which is a laboratory technique, to analyze the levels of expression of SaCas9, MSNs (major neuronal cell type of the striatum), and mutant HTT (Ekman et al., 2019). The following images of immunofluorescence display the differences in gene expression between AAV1-SaCas9-mRosa26 and AAV1-SaCas9-HTT:

Figure 20

In Vivo Disruption of the Mutant HTT Gene in R6/2 Mice (D)



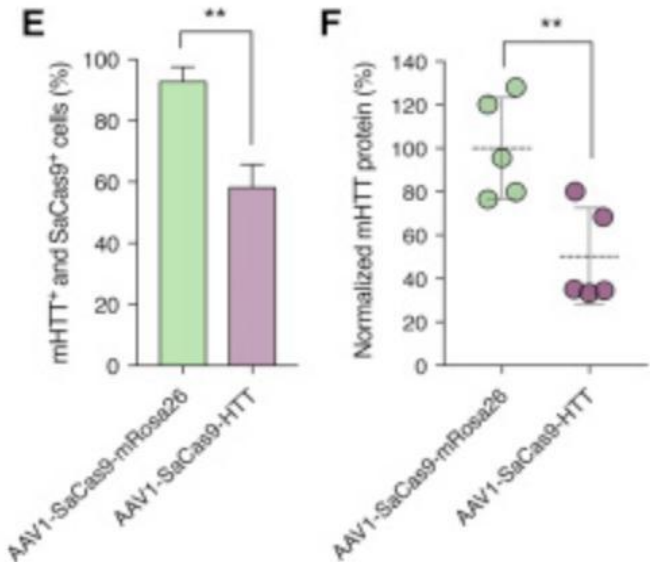
Note: DARPP-32 is a protein involved in neurotransmission and motor control in the brain. The arrowheads indicate DARPP-32+ and SaCas9+ cells with high or low mHTT protein levels.

Source: (Ekman et al., 2019).

These high-resolution images show that the mice that were injected with SaCas9 targeting mHTT had more reduced levels of this protein. Researchers also found that mice injected with AAV1-SaCas9-HTT had 40% fewer mHTT protein levels in DARPP-32 and SaCas9 cells compared to those injected with AAV1-SaCas9-mRosa26. In the measurement of mHTT protein levels, mice injected with AAV1-SaCas9-mRosa26 had mHTT levels 50% higher than those injected with AAV1-SaCas9-mRosa26 (Ekman et al., 2019). The following charts display these results:

Figure 21

In Vivo Disruption of the Mutant HTT Gene in R6/2 Mice (E and F)



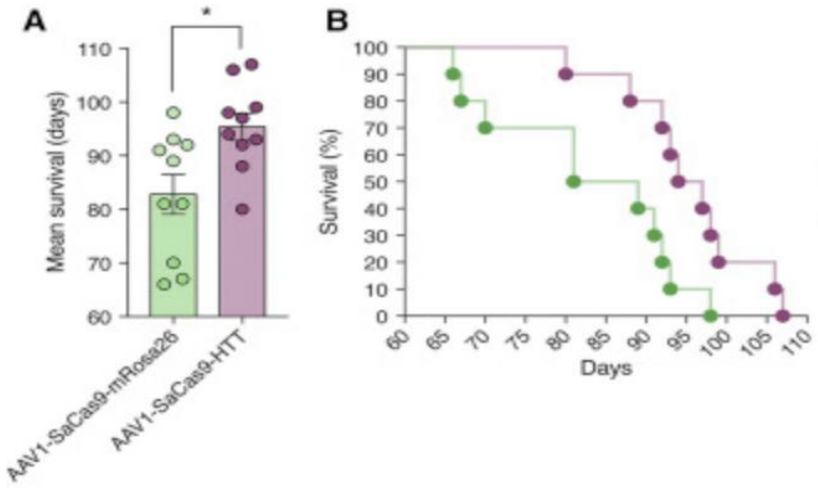
Source: (Ekman et al., 2019)**.**

This quantitative data evidences that SaCas9 intervention is useful for reducing mHTT expression and potentially treating HD.

Finally, researchers measured motor dysfunction, hindlimb clasping (HD symptom), and weight every week in R6/2 mice 4 weeks after AAV injections. Mice injected with AAV1-SaCas9-HTT showed a 15% increase in mean survival compared to mice injected with AAV1-SaCas9-mRosa26. They also had a lifespan of 80-106 days, while the control group lived for 66-98 days (Ekman et al., 2019). The following charts display these results:

Figure 22

CRISPR-Cas9-Mediated Disruption of the Mutant HTT Gene Provides Therapeutic Benefit to R6/2 Mice (A and B)

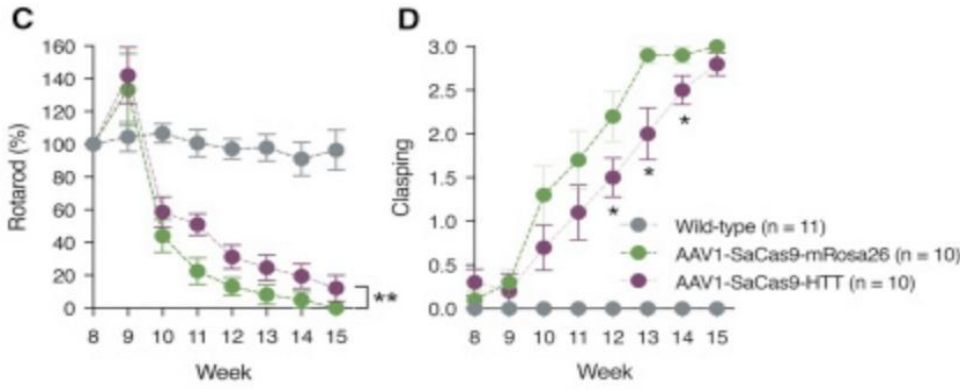


Source: (Ekman et al., 2019)**.**

In addition to this, researchers also observed that R6/2 mice treated with SaCas9 targeting HTT had an improved motor function and a decreased hindlimb clasping at most weeks. More than half of the mice treated by SaCas9 targeting HTT didn't display hindlimb clasping until week 11, while 80% of the mice injected with AAV1-SaCas9-mRosa26 had already clasped by week 10 (Ekman et al., 2019). The following graphs show these results:

Figure 23

CRISPR-Cas9-Mediated Disruption of the Mutant HTT Gene Provides Therapeutic Benefit to R6/2 Mice (C and D)



Source: (Ekman et al., 2019)**.**

In conclusion, these results establish that CRISPR-Cas9 is useful for treating HD at the molecular level and as a treatment for its symptoms in R6/2 mouse models. It reduces the levels of mHTT protein, increases survival rate, and improves motor dysfunction like clasping in the case of mice. This experiment provides positive insights for future applications of CRISPR-Cas9 in the medical field since it proves that this gene editing tool can repress or slow down the development of HD in mice, which, like humans, are mammalian organisms. Even though mice and humans are not completely identical, they have many genetic, biological, and behavioral similarities, making mice useful for the study of disease development and potential therapies.

Applications of Allele-Specific gRNAs to Different HD Patients' Cell Lines

Finally, a study analyzing CRISPR-Cas9 in HD-patient-derived cells will be explained. For this purpose, the article Wan, J., Kim, K., Chao, M., Atwal, R., Gillis, T., MacDonald, M., Gusella, J., Lee, J. (2016) Permanent Inactivation of Huntington's Disease Mutation by Personalized Allele-Specific CRISPR/Cas9 from the journal *Human Molecular Genetics*. The study's focus was on improving allele specificity in CRISPR-Cas9 based on Protospacer Adjacent Motif (PAM) to target HD-patient-specific CRISPR-Cas9 sites.

With this purpose, researchers tested whether different combinations of allele-specific PAM-altering gRNAs could excise the CAG mutation in different HD cell lines *in vitro*. These included a neural precursor cell (NPC) from an iPS cell line (CS97iHD-180n1) derived from the Coriell fibroblast line GM09 and an iPS cell line derived from the Coriell fibroblast line GM04723 (Wan et al., 2016). Researchers tried different types and combinations of gRNAs to target the mHTT gene.

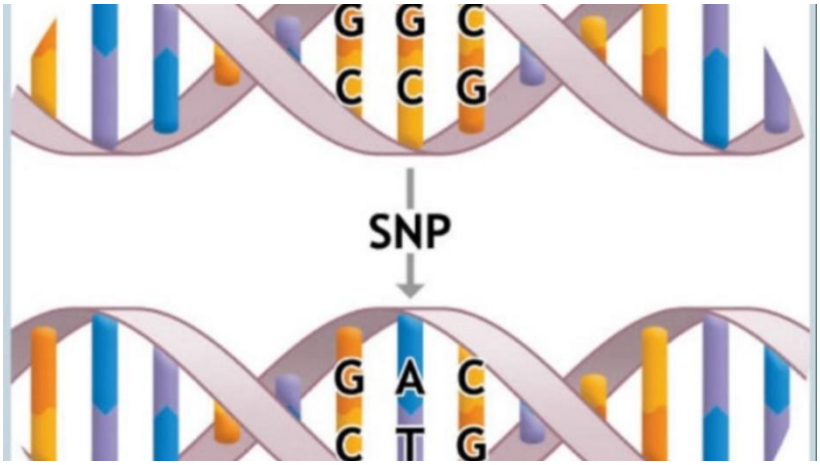
Researchers used two allele-specific gRNAs simultaneously in an NPC line and an iPS cell line. This resulted in the excision of the target region (PAM sequence and mHTT gene), showing that CRISPR-Cas9 was useful. Scientists discovered that approaching HD by directly targeting DNA to only inactivate the mutant allele was effective. They approached the gene-editing method by using a personalized dual gRNA, and this was a useful approach to CRISPR-Cas9 technology.

Researchers also used PAM-altering single nucleotide polymorphisms (SNPs) to directly monitor the specific allele sequence in DNA (Wan et al., 2016). SNPs alter a single nucleotide, and they can be useful to edit the PAM sequence to either bind the Cas9 protein or prevent more cleavage. For example, SNPs can introduce nucleotides to form the sequence NGG, making it a

new potential target for CRISPR-Cas9. On the other hand, SNPs can change nucleotides from the PAM sequence when necessary, such as turning NGG into NGA. This approach was used in the experiment to increase the specificity of the method, and its effectiveness provides useful information about the use of SNPs in CRISPR-Cas9 treatment for HD. The following image displays the functioning of SNPs, which only modify a single nucleotide:

Figure 24

Single Nucleotide Polymorphisms (SNPs)



Source: (Folate, 2023)**.**

This study used human cell lines, which is a significant advancement for the use of CRISPR as a treatment for Huntington's Disease. Using dual gRNAs and SNPs is an effective approach that can increase the success of the treatment in the long term (Wan et al., 2016).

These studies together show that there have been different approaches to investigating CRISPR-Cas9 as a treatment for HD. There have been *in vitro* studies in both mice and human cell lines and *in vivo* studies in mouse models. This research project compiled some of these studies, and, overall, they all evidenced that CRISPR-Cas9 is useful to treat HD. It can target the mHTT gene and the mRNA sequences that regulate it. The *in vitro* studies showed that CRISPR-Cas9 can regulate cellular activities involving the mitochondria and apoptosis, while the *in vivo*

studies indicated that CRISPR-Cas9 can reduce motor dysfunction and increase the lifespan of organisms. These results support that CRISPR-Cas9 is an effective treatment for HD and provides very useful insights for future investigations and applications in the clinical field.

There is also a significant relationship that can be observed between the molecular mechanisms that take place in the neurons at the striatum due to HD and the processes that are repaired through CRISPR-Cas9. For example, neurotoxicity is the most common cellular effect of HD, but the studies evaluated previously showed that this can be repaired through CRISPR-Cas9. One of the investigations also displayed decreased clasping and improved motor function in mice, which correlates with the symptoms of HD. This evidences that CRISPR-Cas9 is a very specific technology that can target the desired site. It is a very practical method to treat HD, and its applications in models must be continued to obtain more insights into its implementation as an HD treatment in the medical field.

Findings

The purpose of interviewing several specialists and asking them questions in these three groups was to obtain different points of view to analyze patterns among their visions and use their responses to solve the initial research question: Is CRISPR-Cas9 gene modification an effective treatment or cure for HD? While there were specialists that answered questions from only one group, others responded for two; this wholly depended on the person's experience in both treating HD patients and researching or studying gene therapy, in this case, CRISPR-Cas9. These specialists were sufficiently reliable to use their responses, compare them with each other, and either approve or refute the hypothesis stated previously in the project. In the following section, the responses of the specialists during the interviews will be displayed, which were later used to analyze their answers in the search for patterns, similarities, or differences. It was divided into three groups of questions—only HD, only CRISPR-Cas9, and CRISPR-Cas9 as a treatment for HD—and the specific questions that were asked in each of them.

Interviews about HD: Lisa Mooney, Dr. Kyle Fink, Dr. Diana Castellanos

The questions about HD were mainly based on the onset of the disease, the severity of its symptoms, the effect it has on the population and the quality of life of both its patients and family members, the treatments available, and the possible cures or new treatments that are being investigated. These questions intended to have the view of HD of specialists that have interacted and worked with patients directly since they have a more pragmatic and reliable perspective on the topic. Analyzing the disease and the current state of its treatments was important to consider the impact that CRISPR-Cas9 can have on HD patients and ultimately answer the research question. The specialists that responded to this group of questions were Lisa Mooney, the social worker of UC Davis HD's Clinic, Dr Kyle Fink, the associate professor of the Neurology

Department and the associate director of the Gene Therapy Center of UC Davis HD's Clinic, and Dr Diana Castellanos, a neurologist in Hospital de Caldas. This section will be divided into the answers to the pre-established questions, which were asked to these three interviewees.

Question 1: How prevalent is HD, and to what extent is it considered common or rare?

Lisa Mooney: HD is an uncommon disease: not many people have it and it's not well known either in the US or in the rest of the world. In 2024, there were 41,000 individuals in the US diagnosed with HD, and this put 150,000 to 200,000 people at risk since the offspring of these HD patients would most probably develop the disease too. There should be more awareness about HD and money available for research, since, this way, probably more people will understand it.

Dr. Kyle Fink: The prevalence of HD is higher than usually reported because many people are not being tested. Many people have "intermediate" CAG repeats (don't reach the minimum for HD development), which affect them but don't manifest into a clinically diagnosed HD. However, it is still considered a rare disorder.

Dr. Diana Castellanos: HD is not a common disease, and it's even considered a rare disease, which refers to one that only affects a small percentage of the population. The frequency of HD is around 8-10 patients per 100,000. Dr. Diana has personally had very few HD patients when compared to other neurodegenerative diseases, such as Alzheimer's (AD) and Parkinson's disease (PD). There's around 1 HD patient per 100 PD patients, highlighting the rarity of the disease. Since HD is so uncommon and complex, it's harder to treat because the resources in hospitals aren't as available to treat it as they are for more common diseases. However, there's a health effort to treat it and there are international resources destined for it.

Question 2: Does HD have a significant impact on the Colombian/US population? (Depends on the nationality of the interviewee).

Lisa Mooney: Yes, HD has a significant impact on the US population. While it is a rare disease, its symptoms and the conditions in which the patient has to live are devastating. It has a notable effect on the US population not because of its frequency, but rather because of its severity.

Dr. Kyle Fink: Yes, HD has a significant impact on the US population because it tends to impact people in the prime of their lives when they would be able to contribute and earn the most. HD has a major burden on the economy and workforce because it usually affects people in the mid to late 40s and early 50s, which is an age when they're productive, and the disease usually prevents patients from continuing to work. Also, taking care of an HD patient is expensive and requires a lot of time, which impacts the family, too.

Dr. Diana Castellanos: HD has a significant impact on the Colombian population; however, its effect isn't related to the frequency of the disease, but rather to the severity of the condition and its symptoms. HD is very complex at the molecular level, and this leads to many physical, cyclical, and behavioral problems for the patient. It also has a deep impact on the family of the patient, which has to take care of the patient almost full-time during the latest stages of the condition. This effect on the community that is perceived in Colombia can be observed in any part of the world.

Question 3: Do you believe that HD has a significant impact on the patient and his/her family?

Lisa Mooney: Yes, there is a very significant impact both on the person and his/her family members. The person is affected in his/her relationships, employment, getting along with

neighbors, and other aspects of their daily life. HD patients don't always need care; in the early stages of the disease, they're still independent: they can drive and talk correctly, for example. However, after 10 or 15 years of the onset of the disease, they start to need more care, and, years later, they'll probably need 24/7 care.

Dr. Kyle Fink: Yes, it has a major impact in both the patient and the family because it prevents the patient from doing activities they did before and demands the family to take care of the patient and spend money. Not only the patient suffers emotional and physical distress, but the family does too. Due to the age of onset of the disease, it's usually the children who take care of the sick parent, making it have a more intense emotional impact on the kids. The family members of the patient change how they're able to live a normal life and have a large psychological impact on wondering if they'll also develop HD later in life.

Dr. Diana Castellanos: HD has a very significant impact on both the patient and his/her family members. HD affects the central nervous system through the formation of protein aggregates, which spread to a high amount of cells in the system, especially those that are guided by the GABA neurotransmitter. This causes excitotoxicity, which is the uncontrolled death of neurons or neurodegeneration, in this case in the central nervous system. The excitotoxicity that takes place during HD is so severe that it could be said that HD is the disease with more implications in the brain. There's a complete destabilization of the mood and psychotic conditions of the patient, and it's as if it combined the symptoms of AD and PD. It's also a progressive disease, meaning that the symptoms are each time more intense until they become completely devastating for the patient. Dr. Castellanos is currently attending an HD patient; she's 25 years old, and Dr. Diana has observed the detrimental effect the disease is having on her: the inability to sleep well, chorea (uncontrolled movements), and other progressive symptoms that

each time become worse. Additionally, the impact of the disease is very hard to control, since the symptom relief is very partial and limited—medications are not that effective. The family also experiences a significant impact because, as the disease progresses, the members have to take full-time care of the patient, which is demanding and time-consuming for them.

Question 4: Which is the first symptom that usually presents in HD patients?

Lisa Mooney: The first symptom in HD patients is usually cognitive changes, difficulties at work, such as in learning new tasks, and mood changes like an increase in anxiety and depression. Not everyone presents chorea, and this symptom usually starts 8-10 years after the onset of the disease—if a patient presents chorea as the first symptom, it means that they already had the disease 5-10 years before that.

Dr. Kyle Fink: The first symptoms that present in HD patients are usually cognitive impairments, irritability, and impulsivity.

Dr. Diana Castellanos: The first symptom that usually presents in HD patients is chorea (uncontrolled movements). This is a pattern that has been observed in HD patients around the world, but it doesn't mean that all patients present chorea as their first symptom or that they even present it throughout the disease. For example, many of the patients who don't start with chorea start with psychiatric symptoms. Chorea in HD is characterized by dystonic postures that may begin in the neck or hands, but then spread to many parts of the body, making it multisegmental. These are uncomfortable positions that can't be controlled easily with toxins. Chorea is also characterized by quick movements when the patient is trying to stay still, and, conversely, slow movements when the patient is trying to move. The uncontrolled movements are not only present as chorea, but they might also present as parkinsonism, which is the movement disorder in PD. Parkinsonism is characterized by rigid positions, tension, and slow and small movements. Some

HD patients have parkinsonism, others have chorea, others have both, and others have none. However, movement disorders like chorea are usually the first symptoms in HD patients.

Question 5: Do all HD patients show similar symptoms and development of the disease, or does this vary?

Lisa Mooney: There are some similarities, especially in those patients that develop the cognitive symptoms first. However, there are three types of symptoms in HD, and not all patients display them at the same levels or some don't even present some of the symptoms at all (it's patient-specific). These are mood and behavior changes, chorea, and cognitive issues, which indicate that the variations in the symptoms are highly correlated with the parts of the brain that are affected. The symptoms of the disease are also affected by the family members of the patient. Family members who motivate HD patients to be more proactive and have a healthy lifestyle and support them will help them to ameliorate the symptoms.

Dr. Kyle Fink: It can drastically vary. Although some cognitive and emotional symptoms usually present first in HD patients, there isn't a definitive pattern of disease development among patients.

Dr. Diana Castellanos: No, not all patients develop the disease in the same way. HD has a triad of symptoms, which are the cognitive, psychiatric/behavioral/emotional, and motor symptoms. Any of the three symptoms can develop first in the patients, and there's only a pattern in many of the patients presenting chorea first, but there's still variation in the development of the disease, which is explained by the fact that HD is multimodal—it affects many parts of the brain at the same time. Additionally, patients have a different age of onset of the disease, depending on the repeats of the CAG codon in the mHTT gene. HD usually develops when the

individual has 40 or more CAG repeats, and, if there are more repeats it means that the age of onset will be smaller. The average age of onset of HD is between 30 and 40 years old.

Question 6: If this varies, what does it depend on?

Lisa Mooney: There's no clarity in the reason for the variation in the development of symptoms in HD patients. As mentioned before, it is patient-specific, and no studies have discovered the reason for this.

Dr. Kyle Fink: No one knows for certain what this variation depends on. It could be because of the family history or the size of the CAG repeats.

Dr. Diana Castellanos: There's not a clear or definite reason for the variation in the development of HD in the patients. This can be generalized to most genetically inherited diseases—they develop differently in each individual, and there still isn't knowledge about why that is. In HD, patients can present the same amount of CAG trinucleotide repeats and still present different symptoms. It might also depend on the specific part of the brain that is affected within the central nervous system in each patient.

Question 7: Which treatments are usually implemented for HD patients?

Lisa Mooney: The main treatment for HD is medication that manages the symptoms, but doesn't cure the disease or slow its progression. Medication is more effective for motor symptoms, like chorea, and psychiatric conditions like mood changes, anxiety, depression, and frustration. Nonetheless, medication for cognitive symptoms is not that useful, which is why most HD patients treat these by getting into a daily routine, using walkers or speech devices, having a strong support system, doing exercise, eating well, going on bike rides, and going to the doctor constantly. For cognitive and psychiatric symptoms, HD patients usually go to a psychiatrist, therapist, or social worker to talk about coping and plan for their future.

Dr. Kyle Fink: Right now, there are approved drugs that reduce chorea and motor disorders and palliative care to address some of the cognitive and emotional symptoms.

Dr. Diana Castellanos: In both Colombia and the rest of the world, the treatment available for HD is medication. One of the main medications that is consumed by HD patients is tetrabenazine, which acts on dopamine to reduce its activity and improve the motor symptoms of the patient like chorea. Tetrabenazine acts over the neurotransmitters in the central nervous system and tries to stabilize all the disorder in the brain. One of the disadvantages of medications for chorea, like tetrabenazine, is that they can have side effects like producing parkinsonism. Other medications include neuroleptics and antipsychotics, which regulate chorea and excitability or irritability, respectively. However, these are known for causing the patient to gain weight. Others include risperidone (psychiatric symptoms) and antidepressants like mirtazapine. Furthermore, valproate is also a used medication to treat the psychiatric symptoms of HD. Other side effects of the medications not mentioned previously include alterations in the liver and gastric issues; these side effects are many of the times caused by the interactions between the drugs when many are consumed at the same time.

Question 8: Are these treatments useful in regulating the symptoms of HD?

Lisa Mooney: Medications can be effective in regulating the symptoms in some cases, but they particularly aren't useful in controlling the struggles in work and relationships of HD patients. Additionally, the treatments are more useful in regulating psychiatric and motor symptoms than cognitive symptoms because of their availability. The efficiency of the medications and other treatments also depends on the attitude of the patient and how they cope: a positive attitude is usually beneficial for ameliorating the symptoms.

Dr. Kyle Fink: They can somewhat be successful in treating the symptoms, but not completely. Sometimes they can be effective, but they usually don't ameliorate the symptoms as expected, and there isn't a significant positive impact on the patient.

Dr. Diana Castellanos: The medications mentioned in the last question can attenuate the symptoms, but there isn't a significant relief as it can be seen with treatments in other diseases, such as PD. The relief of the symptoms is very partial and limited.

Question 9: Has any definitive cure for HD been discovered, or is it in process?

Lisa Mooney: Researchers are currently looking at CRISPR-Cas9, stem cell technology, and Antisense Oligonucleotides (ASOs) for the possibility of a treatment, but none of them look like they're going to cure the disease.

Dr. Kyle Fink: Cures for HD are still in progress; they are hard to achieve, but there are many promising treatments underway. The promising treatments are generally related to interference in the central dogma of biology, which is going from DNA to RNA and then to protein, in the mHTT gene specifically. Some of the promising treatments include those targeting the mHTT protein aggregates, the mHTT RNA through Antisense Oligonucleotides (ASOs) or short interfering RNA (siRNA), the mHTT gene through CRISPR-Cas9, or the expression of the gene through epigenetics.

Dr. Diana Castellanos: There are medications for HD that are still not available in Colombia, such as variants of tetrabenazine that have fewer side effects than the original: they don't cause dizziness or parkinsonism. Apart from this medication, there are not more that could be brought to Colombia, since the rest are already in the country since they've been developed between the last 10 and 20 years. At the clinical trial level, one of the treatments or possible cures that has been proposed since several years ago is the edition of the genome through

molecular techniques that are each time more optimized and specialized. This can't be done with Alzheimer's and Parkinson's disease, for example, because they depend on many genes and not only one, like HD: they're polygenic and multifactorial diseases. Since HD is only caused by one mutated gene, it's very possible that cutting that section through gene-editing techniques like CRISPR-Cas9 can be useful and later implemented as a treatment. It's not in action right now, but it's in the process through research with mice and some humans, even though it has been notably risky because of the difficulties of controlling DNA. CRISPR-Cas9 experimentations have been controversial for this reason, like in the case of one that was done on some girls in Japan to prevent Human Immunodeficiency Virus (HIV) development. The experiment epitomized that the treatment couldn't be done in all the cells of the body and that it was uncontrolled, which could cause mutations in other cells.

Question 10: In case there's an HD cure in process, do you believe it will be successful?

Lisa Mooney: The treatments that are in process, which were mentioned previously, will probably contribute to regulating the symptoms, but not cure the disease completely. Specifically, these technologies will delay the onset of the disease (the disease won't show up at such a young age)—this is called disease-modifying. HD is so complex because of the different parts of the brain that it affects, and this makes finding a definitive cure for it little possible.

Dr. Kyle Fink: A successful treatment and cure for HD is on the horizon; the tools that researchers have to implement those potential treatments are getting better every day. The technology currently available to treat disease, especially through techniques that alter the central dogma of biology, is at an unprecedented level and there's huge potential in it.

Dr. Diana Castellanos: The HD cure in process, which might be gene-editing techniques to remove the mutant gene that is causing the disease, can be successful theoretically. However, this still can't be confirmed definitely because the technology is still in the process of research and hasn't been implemented to any HD patient.

Interviews about CRISPR-Cas9: Dr. Jhon Fredy Betancur, Dr. Natalia Garcia

The questions asked about CRISPR-Cas9 were mainly based on the efficiency of the method, its margin of error or risks, the past applications of the technology, and its potential to treat genetic diseases. The purpose of this set of questions was to have testimonies or opinions of specialists who have studied CRISPR-Cas9 in depth or have researched or experimented with it since this provides them with the knowledge to analyze the technology as a whole, and this was a crucial part to respond to the research question of the paper. This section will be divided into the answers to the pre-established questions, which were asked to all the interviewees.

Question 1: Do you believe that CRISPR-Cas9 is an efficient technology to modify the genome?

Dr. Jhon Fredy Betancur: Yes, CRISPR is an efficient method to modify the genome. That's the sole purpose of the technology, to edit a gene or section of DNA with a guide RNA that is complementary to the DNA sequence. The procedure of CRISPR is very efficient when it's well set, organized, and precise.

Dr. Natalia Garcia: Yes, CRISPR can be considered an efficient technology to modify the genome, especially in terms of its specificity when targeting a gene. Gene modifying technologies have massively evolved throughout the years; for example, the methods in the 70s or 80s were little efficient because there weren't constant results, they were very expensive, and many devices or materials were required. Some of the technologies during that time included

Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs). Nowadays, CRISPR is more viable and effective than the previous techniques, mainly because of the increasing research and knowledge about bacterial defense mechanisms and how CRISPR developed from that.

Question 2: Do you believe that CRISPR-Cas9 has a high error margin or is very risky?

Dr. Jhon Fredy Betancur: No, there are very few risks with CRISPR because the sgRNA is designed to have a perfect homology for an exclusive DNA segment. The only risk that exists with CRISPR is when the gene that is being targeted has a homologous section (a section with the same or a similar sequence of nucleotides). There is the possibility that the sgRNA will bind to the homologous gene and not the official gene that was targeted, and this will not allow the intended gene to be modified and thus prevent the objective from being reached. However, this can be prevented with specificity while designing the sgRNA and then injecting it into the appropriate area of the bacteria, plant, body, or any organism that is being genetically modified.

Dr. Natalia Garcia: Even though CRISPR is highly specific and has little possibility of going to other parts of the genome, there's a notable risk regarding the limited knowledge that doctors have about genomic interactions. Specialists don't know specifically what might happen in the host's genome after CRISPR is implemented. For example, it could affect the proper functioning of somatic or sexual cells, and the little information available about these adverse effects shows a significant risk of CRISPR.

Question 3: Has the CRISPR-Cas9 technology been implemented in the medical field up until today? If not, in which fields is CRISPR-Cas9 used during actuality?

Dr. Jhon Fredy Betancur: He doesn't have precise knowledge of implementations of CRISPR in the medical field because in his job he uses CRISPR as a means to modify the genomes of bacteria or plants, not humans. However, he believes that CRISPR has been used to treat diseases in the United States and that there is currently extensive research on this application of CRISPR in many countries around the world, including Colombia. He doesn't know of implementations of CRISPR for diseases directly in human patients inside Colombia, though. He also highlighted that the use of CRISPR in humans is highly controversial because it implies ethics debates. He does know that CRISPR is highly used in the modification of plant and microbial genomes.

Dr. Natalia Garcia: The use of CRISPR during actuality is mainly present in research *in vitro* (cultured cells) and *in vivo* (mouse models) to develop treatments for diseases. Its actual use in therapeutics hasn't been implemented yet, meaning that it's not common for humans to be genetically modified through CRISPR to treat diseases. The future use of CRISPR in the medical field depends on how it's applied to pharmacogenetics, and there's also a therapeutic alternative where RNA sequences would be modified instead of DNA sequences, either through microRNAs or Cas proteins. In addition to its use in the medical field, CRISPR is also implemented in animals, to improve milk production in cows and in general the condition of livestock. It's also used to modify plants to improve crop fertility.

Question 4: If CRISPR-Cas9 has been used in the medical field, do you believe it has worked or been used effectively? Why?

Dr. Jhon Fredy Betancur: He doesn't know about the use of CRISPR in the medical field specifically, but he does know about its use in cell models in laboratories, bacteria, and plants. For example, it has been used to silence or activate genes in bacteria or in plants to make them more fruitful. These have usually been successful and effective, but this is an experimental technique, meaning that it's subject to mistakes that scientists learn from to change their research methods or approaches. The effectiveness also depends on the gene that is being targeted; those that have more homologous sequences are more challenging to bind to and cut.

Dr. Natalia Garcia: Many researchers are working on this topic around the world. However, while there are publications available about advancements in CRISPR research, those in the clinical practice are very limited because of ethical dilemmas and controversy. This derived from a case in China where two girls were genetically modified as a means to prevent Human Immunodeficiency Virus (HIV), but it caused controversy and ethical dilemmas. Therefore, the use of CRISPR in actual patients is very limited and it still can't be said if it's effective or not. Nevertheless, there have been successful outcomes in *in vivo* models (mice) for different genetic diseases, such as muscular dystrophy or chorea in HD. These are promising and provide insights for later applications.

Question 5: Do you believe that CRISPR-Cas9 has the potential to cure genetic diseases? Why?

Dr. Jhon Fredy Betancur: Yes, CRISPR is promising as a cure for genetic diseases, and previous research has shown that it can do so; it's the objective and projection of the technology for the future. It especially has the potential for rare diseases that are caused by a specific gene.

Dr. Natalia Garcia: Not completely. There's still a lot that specialists don't know about, and the technology still requires more research and studies to prove its possible success in the medical field. Analyzing the use of CRISPR-Cas9 as a cure for a genetic disease is a very broad and complex discussion. It was even admitted in the Nobel Prize of Chemistry of 2020 that the gene-editing technique must be applied carefully because there's still limited knowledge, so it mustn't be implemented into embryos or humans immediately. For this reason, she believes that the technology will treat the diseases, but not cure them completely.

Question 6: Up until today, has CRISPR-Cas9 been used to modify a human genome with the purpose of treating a disease?

Dr. Jhon Fredy Betancur: His research is focused on applying CRISPR to the genomes of plants and bacteria, so he hasn't directly experimented with the medical field. However, he believes that CRISPR has been applied to human genomes—not in Colombia, but in other countries that are more technologically advanced, like the US. Specifically, there's a case in which CRISPR was used to modify the human genome in China. However, it wasn't done without a specific purpose, making it controversial ethically. It is more ethical to research with cultured human cells than with embryos or humans.

Dr. Natalia Garcia: To her knowledge, CRISPR-Cas9 hasn't been used in humans to cure a disease. She believes that there are small possibilities of this, but maybe there have been applications to humans that haven't been published because of the ethical implications that it has. However, there are current *in vitro* and *in vivo* studies (especially in mice) that will hopefully provide useful discoveries for the future use of CRISPR in a human being.

Question 7: Do you believe that the experiments that have been done with CRISPR-Cas9 have been successful?

Dr. Jhon Fredy Betancur: In his area of expertise, which is CRISPR in microorganisms and plants, it has been successful because it has targeted the gene of interest with exactitude and modified the designated genome. He isn't sure about its success when applied to the medical field, but he believes it has a lot of potential in this area and that it's the technology's objective.

Dr. Natalia Garcia: This can usually be determined with scientific papers or other publications about *in vivo* and *in vitro* studies of CRISPR-Cas9 applications. For example, if a mouse model is being used to test the efficiency of the technology to treat a disease, and improvements in the production or depletion of a protein, gene, receptor, or another molecule that is being affected by the disease are observed, then it can be determined that the experimentation was successful. It depends on the outcomes of the experimentations and the models in which they're being done.

Question 8: Do you believe that the amount of times that CRISPR-Cas9 has been experimented are enough to trust on implementing the technology in the medical field on a larger scale?

Dr. Jhon Fredy Betancur: Yes, this is a very advanced and efficient technology that is very precise with the genome, so it can be trusted in the future for the medical field if the necessary research is done previously. A fact that allows scientists to trust this technology is the Nobel Prize in Chemistry that was given to Emmanuelle Charpentier and Jennifer Doudna in 2020. This proves that it's a very significant advancement with great potential for the future of medicine.

Dr. Natalia Garcia: No, specialists still can't trust the technology completely because there are still many unknown concepts about it. More research is necessary to start implementing the technology in human patients, and, after this, it can be applied on a larger scale. Implementing the technology on a larger scale right now would be very dangerous and could bring unexpected outcomes for the patients, such as cancer development and malfunctioning in sexual or somatic cells.

Question 9: Do you believe that the successful outcomes of experiments with CRISPR-Cas9 in cultured cells and mouse models support the fact that it could be successful in treating a human disease?

Dr. Jhon Fredy Betancur: Yes, CRISPR is constantly being studied through research, especially through cultured cells and mice when applied to the medical field. There is progress on this technology every day, which means that these studies will support the use of CRISPR in human patients in the future. Also, cultured cells and mice work similarly to humans—they're useful models to imitate people.

Dr. Natalia Garcia: Scientists who lead research in cultured cells and mouse models usually defend that the results are promising. If mouse models are used to analyze a genetic disease and the results show that there's less progression in the disease, then it could be said that the outcome is successful and that it supports future use in humans. However, transitioning from a mouse to a human is complex and the success of these models doesn't completely ensure the future success of the technology. One of the main challenges will be the reproducibility of CRISPR-Cas9 in the cells of the organism, and this highly depends on the type of cells that are being intervened.

Question 10: Has CRISPR-Cas9 been used in Colombia/US? If yes, for what? Has it been used to treat diseases? (Depends on the nationality of the interviewee).

Dr. Jhon Fredy Betancur: CRISPR is probably being researched in Colombia for medical purposes through cell and animal models, but it hasn't been used in a human patient. Nevertheless, he has seen that CRISPR is used more in Colombia to modify the genomes of microorganisms or plants. For example, there have been projects where CRISPR has been used to edit the genes of chlorophyll in plants, to improve photosynthesis or disease resistance. It's easier to work with microorganisms and plants than humans because their organisms are less complex and they bring less ethical controversy.

Dr. Natalia Garcia: She doesn't know about the use of CRISPR-Cas9 to treat diseases in Colombia. As she said before, there haven't been publications about the use of CRISPR in humans, but she believes that there is *in vivo* and *in vitro* research in Colombia to progress in the use of the technique.

Question 11: Do you believe that CRISPR is a viable option to treat genetic diseases in terms of its cost and necessary materials?

Dr. Jhon Fredy Betancur: CRISPR would be very expensive as a treatment for monogenic diseases, and especially for rare illnesses, because it's a technology that requires many experiments in laboratories with cells or animal models, which implies spending a significant amount of money on research and development, and the price of CRISPR must compensate for these costs. However, CRISPR won't be as expensive if it is mechanized or standardized in clinical centers in the future; it can even be less expensive than the ordinary treatment of genetic diseases. It will be an investment that's worth it because it saves time and

money that will be invested later in other treatments, medications, appointments, palliative care, or therapy that won't be necessary if there's an initial CRISPR treatment that is successful.

Dr. Natalia Garcia: The plan for the future use of CRISPR-Cas9 is to treat genetic diseases. The technology is efficient and it has the potential to do this, but several considerations must be taken into account before. Humans are complex organisms and specialists don't know about the whole functioning of DNA; for example, there are variations in the epigenetics of different individuals and their development of symptoms, as in Down Syndrome, and scientists still don't understand this completely. The specific mechanisms that take place during each genetic disease must be taken into account; for example, it must be considered if it affects the production of an enzyme or a receptor. Another complication in terms of available materials and costs is passing from *in vivo* and *in vitro* studies to human beings; it's more complex. The application of CRISPR could also bring side effects and have the risk of not being as successful as imagined: gene variants and mutations will always continue to appear because humans are constantly evolving, so it's very hard to control the genome.

Interviews about CRISPR-Cas9 as a Treatment for HD: Dr. Kyle Fink, Dr. Diana Castellanos, Dr. Natalia Garcia

The questions about CRISPR-Cas9 as a treatment for HD were focused on the efficiency of the method as a means to treat or cure the disease. The purpose of this group of questions was to analyze the answers of specialists who know about HD or have treated HD patients, and, by that, have studied the advantages or disadvantages of using CRISPR-Cas9 as a treatment. This was the most important group of questions to respond to the research question that was set at the beginning of the project. This section will be divided into the answers to the pre-established questions, which were asked to all the interviewees.

Question 1: Do you believe that CRISPR-Cas9 is a viable option to treat HD?

Dr. Kyle Fink: Yes, CRISPR-Cas9 is a perfect candidate as a gene-editing technique to treat genetic diseases like HD because it can target the specific gene that is causing the disease and cut it. Although it is a viable option, the questions that arise are how the treatment would be developed and how it can be compared with other treatment options.

Dr. Diana Castellanos: Yes, CRISPR-Cas9 could be a viable option to treat HD, but the point is that it would have to be done in all the cells of the organism, or, in this case, the brain cells. Spreading CRISPR through the body cells makes the treatment complex, and a lot of time is needed for specialists to find a way to do so. Many pluripotent stem cells are being used for this purpose, but there's a lot of mutability, and little control, and this causes destabilizations in other genes. If the factors mentioned previously are worked on and improved, then CRISPR-Cas9 can be a viable option to treat HD, but there's still a lot of research needed to reach that. It's also important to highlight that this treatment is more viable if it's applied in pre-diagnosed patients that still haven't developed the disease than if it's done in patients that already have the disease developed since they are more vulnerable to the risks of the technology. The potential in HD is that it can be pre-diagnosed by counting the CAG repeats in the mHTT gene: if there are 40 or more repeats, the person will develop HD in the future. It's also easy to observe this since the disease is monogenic—it only occurs because of one mutation. HD is also the perfect disease model for CRISPR-Cas9 because the effects of the disease are so devastating that for most of the patients, it's preferable to live with the side effects or mutations caused by CRISPR-Cas9 than to live with the disease. Dr. Castellanos did mention that knowledge of gene-editing techniques has increased meaningfully during the last few years, but these still have to be studied in more depth.

Dr. Natalia Garcia: During actuality, many research projects are focused on the use of CRISPR-Cas9 as a treatment for HD because the molecular mechanisms of the disease make it a great model for the technology: its extended trinucleotide repeats could be edited through CRISPR-Cas9. Many multicentric studies are working on this and HD patients would like to participate in the testing of the technique. Therefore, CRISPR can be a viable option to treat HD, but it's still in the process of investigation.

Question 2: Do you believe that CRISPR-Cas9 could regulate the symptoms of HD, such as chorea?

Dr. Kyle Fink: Yes, the hereditary nature of HD makes it so that CRISPR-Cas9 can regulate any symptom of the disease, including chorea. If the therapy is well done and the intervention takes place at the right time, then the symptoms will be regulated correctly. The right moment to use the CRISPR-Cas9 treatment in HD patients would be 10-15 years before the onset of the disease and before the cognitive and mood changes arise. The treatment will be more effective if it's done earlier in the disease progression than later.

Dr. Diana Castellanos: In CRISPR-Cas9 it would be very complex to regulate a specific symptom; instead, there would be a regulation of the disease as a whole. Right now, however, the progression of the symptoms would probably be very slow or minimal—in more time, the impact on the symptoms will probably be more significant. The regulation of the symptoms will also work in relation to the time in the disease that the treatment is done. It will be more efficient if CRISPR-Cas9 is done in pre-diagnosed patients, in the early stages of their life, or when the disease is still not that aggressive. This last scenario is possible when there are less than 40 repeats of the CAG trinucleotide since this makes the presentation of the disease late and mild.

Dr. Natalia Garcia: Yes, the idea is that CRISPR-Cas9 will help in ameliorating the symptoms of HD, but the efficiency in regulating symptoms like chorea depends on the progression of the disease and the moment in which it's intervened through the gene-editing technique. The regulation of symptoms like chorea will be more successful when CRISPR is done before the onset of the disease or early in its progression. However, treating the symptoms will be more difficult if CRISPR is done in a late stage of the disease, where the protein aggregates are already present and it's hard to regulate the effect that these have on neurotoxicity, for example. It all depends on the harm that has already been caused to the brain. If CRISPR-Cas9 is implemented before the progression of the disease, a new system of diagnostics would have to be established. There would have to be a technology where the genomes of newborns can be sequenced to search for extended CAG repeats.

Question 3: Do you believe that CRISPR-Cas9 can be a definitive cure for HD in the future?

Dr. Kyle Fink: Yes, CRISPR-Cas9 could be a definitive cure for HD, but this depends on how it's used and implemented on HD patients. If researchers can target just the toxic strand of the mHTT gene in an early stage, then it can cure the disease. However, CRISPR-Cas9 isn't the best option to cure HD, since other options target the mutant RNA or the protein aggregates formed, which are the ones that directly impact the central nervous system. These other options can stabilize, remove, or decrease the toxicity of the mHTT proteins. Conversely, the treatments that target RNA directly include antisense oligonucleotides (ASOs) and small interfering RNAs (siRNA). Finally, another option of a cure for HD that is an alternative to CRISPR-Cas9 is the regulation of the mHTT gene so that it's not expressed through the field of epigenetics. For the cure options for HD, it's important to think about how the central dogma of biology can be

targeted: DNA (through CRISPR-Cas9), RNA, and protein. Even though there are alternatives, CRISPR-Cas9 can be a definitive cure because it can delete the additional CAG repeats that cause the disease, and this would regulate the mitochondrial stress, reactive oxygen species (ROS), inflammation, and energy biosynthesis failure that is caused in HD, and which can't be ameliorated through supplements and other therapeutics.

Dr. Diana Castellanos: This cannot be defined at the moment; it depends on how the research on the topic progresses throughout time and if the problems mentioned previously regarding the risks of CRISPR-Cas9 are solved. Right now, it can be better interpreted as a new treatment rather than a cure, but it's possible that, with time, it can be a cure for the disease.

Dr. Natalia Garcia: The concept of cure is very broad and complex. Curing a disease would require that the appearance of the CAG repeats in the human genomes decreases significantly; this would be complicated because eggs and sperm cells would have to be genetically modified so that the following generations don't carry the mHTT gene, taking into account that, with each generation, the CAG repeats increase in amount. Even if the CAG repeats are depleted, there's the possibility that new mutations will arise because of genomic evolution. New mutations are related to DNA replication, so curing the disease completely would require intervening in this process, which is, again, very complex because it's highly specific and takes place in all cells. The real efficiency of the method is found in the success of testing in models, rather than looking at the technology as a cure, since it will probably treat the disease, not cure it.

Question 4: Do you believe that it would be complex to implement CRISPR-Cas9 as a treatment for HD in medical centers around the world in terms of necessary materials and effectiveness?

Dr. Kyle Fink: Yes, it would be very complex. In laboratories, researchers have access to cells from HD patients and HD mouse models and can manipulate them. However, it's more difficult when implemented for actual HD patients, and one of the main challenges is how it's delivered to the patients. It's important to find a way that is safe and efficient to deliver the CRISPR-Cas9 complex to the central nervous system, which is difficult to reach and to get it to many cells for it to have an effect. Additionally, Cas9 is a large and stubborn protein, which makes it complex to deliver. Some options of methods to inject CRISPR-Cas9 into the cells include AAV, lipid nanoparticles, modeled cells, viral-cell particles, and the ribonucleotide protein complex. However, the main challenge is that the brain is the hardest organ to get into; it's very complex structurally and covered by the skull and blood vessels. There still isn't a mechanized safe way in which this delivery of CRISPR-Cas9 materials can be done to the affected neurons. Nonetheless, there's a hint that CRISPR-Cas9 can be accepted for HD in the future, which is the approval of CRISPR as a treatment for sickle cell disease in December 2023 by the Food and Drug Administration (FDA). Currently, many sickle cell disease patients get their affected cells removed, CRISPR-Cas9 is injected into them, and the healthy cells are implanted again into the patient. The use of this technology within medical centers suggests that it can probably be used in the future for other genetic diseases, such as HD.

Dr. Diana Castellanos: Yes, it would certainly be complex because, even though the research on CRISPR-Cas9 is not as expensive as for other techniques or technologies, it's very expensive to implement in clinical centers, and Colombia particularly has a severely affected

health system. It's relatively accessible, but at this moment it's very complex for Latin America to pay for access to medications, so it'll be even more difficult to pay for treatments like CRISPR-Cas9. The treatment would first be implemented in the United States and Europe, but, it would be a challenge to take it to Latin America and Africa, who will struggle to pay for it. In Colombia, there's a deficit in the EPS and many of the decisions that institutions make are limited by EPS; therefore, other mechanisms should be created so that more countries can have access to medications and treatments, such as international alliances.

Dr. Natalia Garcia: Yes, this will be very complex, and more research is required for it. The current phase of this topic includes multicentric studies and the "recruitment" of HD patients to start testing the technology. The purpose of these tests is to perceive and analyze the evolution in the patients and the possible risks of implementing the technique on a large scale. One of the risks, for example, includes cancer development because, if CRISPR-Cas9 targets unintended sequences, it can cause molecular alterations that will lead to the uncontrolled proliferation of cells.

Question 5: Do you believe that it would be complex to implement this treatment in Colombia/US? (Depends on the nationality of the interviewee).

Dr. Kyle Fink: Yes, it would be complex to implement this treatment in the US. The technology is very novel and challenging, and it requires specialized facilities. The cost of manufacture is high because this gene-editing technique takes years and millions of dollars to develop; therefore, few people will have the opportunity to pay for CRISPR-Cas9. Some centers try to make gene-editing techniques affordable, but any novel treatment like CRISPR will automatically be expensive. For example, the treatment of CRISPR-Cas9 for sickle cell disease, also known as Casgevy, costs around 4 million dollars, and this indicates that CRISPR-Cas9 for

HD will probably be within this range. This will make it difficult for the technology to be implemented in the US.

Dr. Diana Castellanos: Yes, it would be complex to implement this treatment in Colombia because there aren't enough resources to pay for the implementation of CRISPR-Cas9 in medical centers. Additionally, it would be hard to have access to it when other countries are selling it or have used it with more frequency (probably the US and Europe) since the EPS limits the decisions that Colombians can make regarding the buying of medications and treatments outside of the country.

Dr. Natalia Garcia: Yes, it would certainly be complex to implement this treatment in Colombia, especially because most of the research projects and papers that are being written about CRISPR as a treatment for HD come from the US, Europe, and Asia. The research on this topic is predominant in those places and not as present in Colombia. It would be challenging to bring this technology (as a treatment for HD) to Colombia, mainly in terms of costs and necessary materials.

Question 6: Are there significant risks for HD patients when treating them with CRISPR-Cas9?

Dr. Kyle Fink: Yes, cutting DNA and inducing Double Strand Breaks (DSBs) pose risks and can cause gene disruptions. It's hard to target only one gene without affecting the surrounding ones, and this can result in errors like an indel (incorporation or removal of one or more out-of-place nucleotides), base-pair alterations, and chromosomic distribution effects like translocation (a piece of one chromosome attaches to another chromosome) or duplication (additional copies of a chromosomal region are produced). While there are some mathematical tricks to design a CRISPR-Cas9 complex that targets the mHTT gene, there are other genes in

the human genome that contain CAG repeats, and there's the possibility that these will be targeted instead of the mHTT gene. These can ultimately cause mutations that can have side effects on the HD patient. Additionally, when DSBs aren't repaired, they can cause cancerous cells, which reproduce uncontrollably. Tumor protein P53 is in charge of helping cure DSBs and preventing cancer development, but it represses the efficacy of CRISPR-Cas9 treatment, so many researchers have been trying to target P53 and silence it as a means of doing CRISPR-Cas9 therapy. This displays the challenge of balancing CRISPR-Cas9 efficiency with controlled tumor risks. However, researchers in laboratories believe that the benefit of CRISPR-Cas9 for HD will outweigh the risks because HD is a devastating disease.

Dr. Diana Castellanos: Yes, the risks that exist for HD patients when being treated with CRISPR-Cas9 include side effects and new mutations. However, there could be other unknown risks that will be discovered at the moment that the treatment is implemented on a larger scale. The treatment may destabilize the genome of the host, cause arrhythmia, or develop cancer, but again, this depends on the reaction that each patient has to it. It's necessary to try CRISPR-Cas9 on thousands of individuals to accurately determine what happens, sometimes without understanding the mechanisms that cause it. Nevertheless, something that makes the technology less risky for HD patients is that it's possibly worse to live with the disease than to confront the risks of the technology—the risks are not as relevant when compared to the disease. In the case of the girls in Japan who were genetically modified with CRISPR-Cas9 to prevent the development of HIV, the risks were more notable because it was a prevention case, so the risks were relevant when compared to the state of the girls (healthy). However, this wouldn't happen in HD because of the reasons mentioned previously.

Dr. Natalia Garcia: The main risk of using CRISPR-Cas9 to treat HD is that CRISPR might end up affecting other genes and causing unwanted or unexpected side effects. While these side effects could be little notable, they could also be severe and end up threatening the patient's life. One significant risk, as mentioned before, is cancer development. Some side effects that could present, for example, are hair loss.

Question 7: Have there been experimentations with CRISPR-Cas9 *in vivo* or *in vitro* in Colombia/US up until today? (Depends on the nationality of the interviewee).

Dr. Kyle Fink: In the US, there have been both *in vivo* and *in vitro* experiments to study CRISPR-Cas9 as a treatment for HD. The *in vivo* studies have mainly included mouse models that have the mHTT gene, while the *in vitro* studies have principally incorporated HD patient cultured cells.

Dr. Diana Castellanos: To her knowledge, there may have been studies in animal models, mainly mice, in laboratories, but she's sure that there hasn't been any application beyond that. Dr. Diana doesn't have knowledge about the success of these *in vivo* and *in vitro* experiments in Colombia. Most of the laboratories that are working on gene-editing techniques are located in the US and Europe, and, if there's any in Latin America that is advanced on the topic, it's probably located in another country (not Colombia), such as Argentina, for example.

Dr. Natalia Garcia: She doesn't know about specific experimentations with CRISPR *in vivo* and *in vitro*, but she believes that studies are being done either in cultured cells or mouse models. As she said before, most research on this topic is done in other countries and is communicated later to Colombia.

Question 8: Has CRISPR-Cas9 already been implemented as a treatment for an HD patient? If yes, was it successful?

Dr. Kyle Fink: No, CRISPR-Cas9 still hasn't been implemented as a treatment for an HD patient in any part of the world. The time that it might take to accomplish this is uncertain, although some laboratories suggest that it will be accomplished in two or three years from now.

Dr. Diana Castellanos: No, CRISPR-Cas9 still hasn't been implemented in an HD patient in any part of the world, but it has been studied in animal and cell models.

Dr. Natalia Garcia: No, CRISPR-Cas9 still hasn't been implemented as a treatment for an HD patient, but it hopefully will be in the future.

Question 9: Has CRISPR-Cas9 been implemented as a treatment for an HD patient in Colombia/US? (Depends on the nationality of the interviewee).

Dr. Kyle Fink: No, it hasn't been implemented as a treatment for an HD patient in the US.

Dr. Diana Castellanos: No, it hasn't been implemented as a treatment for an HD patient in Colombia.

Dr. Natalia Garcia: No, it hasn't been implemented as a treatment for an HD patient in the US.

Conclusions

The answers in the interviews of the three different groups of questions were analyzed within the same categories and compared with the others to ultimately analyze the efficiency of implementing CRISPR-Cas9 as a treatment for HD and respond to the initial research question. The responses of the interviewees in the questions only about HD and only about CRISPR-Cas9 were contrasted more generally since these were not the main focus of the research question and rather worked to support the analysis of the main topic. On the other hand, the responses to each question in the category of CRISPR-Cas9 as a treatment for HD were analyzed individually and more thoroughly, to directly address the principal inquiry of the project. All of the answers were also compared with the findings from the theoretical framework section.

HD: A Devastating Disorder with the Profile for Molecular Therapies

The interviewees who responded to the HD questions highlighted that this is a progressive disease where the symptoms are devastating to both the patient and his/her family members. They said that HD is a rare disorder and that its impact on the population is given by the severity of its symptoms, not by its frequency. Additionally, they underscored that HD symptoms divide into three groups—cognitive, behavioral/psychiatric, and motor—and that the onset of them in each patient varies. They agreed that the disease causes damage in the central nervous system, especially through the formation of protein aggregates that alter important functions—these usually cause mitochondrial dysfunction and lead to excessive apoptosis cell deaths in the brain, which develops into neurodegeneration. The three groups in which HD symptoms divide, as well as the molecular effects, like the formation of protein aggregates and excitotoxicity, were also mentioned in the theoretical framework and obtained from the paper called CRISPR/Cas9 Mediated Therapeutic Approach in Huntington's Disease from the journal

Molecular Biology (Alkanli et al., 2022). However, the interviewees responded differently to the question about the first symptom that arises in HD patients: Lisa Mooney said that the first effects were cognitive, such as struggles in work, while Dr. Diana Castellanos stated that the first symptoms are usually motor, such as chorea and parkinsonism. On the other hand, Dr. Kyle Fink did agree with Lisa in the presentation of cognitive symptoms in the early stages of the disease, but he added that emotional effects like irritability also show up during this stage. In the theoretical studies of the project, it was found that psychiatric and cognitive symptoms are usually present long before the onset of motor dysfunction like chorea (R.A., 2010), which agrees with Dr. Kyle Fink and Lisa Mooney but contradicts Dr. Diana Castellanos. The responses of the interviewees agreed again when they all said that, right now, there's no clear explanation discovered by research that justifies the variation of the presentation of symptoms throughout the disease; some of the interviewees hypothesized that this variation is related to the number of CAG repeats, but no one has discovered the real reason for it.

They also agreed with the fact that the current treatments applied for HD patients are medications and palliative care (care from the family or going to the psychiatrist). It was also found in the theoretical framework that one of the only currently existing treatment approaches for HD is medication; some of the medications mentioned in the theoretical section were also mentioned by Dr. Diana Castellanos, including tetrabenazine (Alkanli et al., 2022). While these treatments are intended to regulate the symptoms and improve the quality of life of the patient, they're very ineffective and don't have a meaningful impact on the patient, which allows this disease to be progressive and to show little improvement throughout time. Additionally, there's not a definitive cure at the moment, which makes HD a bigger issue in the medical field. The fact that HD can be treated, but still doesn't have a cure, was also found in the theoretical framework

(Alkanli et al., 2022). However, the three interviewees agreed that a possible cure or more efficient treatment that might be developed for the future would involve gene-, RNA-, or protein-editing techniques, like CRISPR-Cas9, ASOs, siRNAs, or epigenetics. These don't focus on just a symptom of the disease, but rather focus on the root of the disorder, taking into account that it's a genetic disease. Lisa Mooney believes that these technologies will only delay the onset of the symptoms, but Dr. Diana Castellanos and Dr. Kyle Fink trust that these techniques have a bright future as cures. While these treatment or cure options pose risks, these interviewees highlighted that HD is a genetic disease with the perfect profile to be treated through molecular therapies—it has devastating effects on the quality of life of the patient, which makes a beneficial treatment necessary, and the risks of these technologies aren't that meaningful when compared with the combined cognitive, emotional, and motor symptoms of HD. The exhaustive lists of symptoms that can be found in HD patients, as mentioned in the theoretical framework, display the devastating conditions in which HD patients live (R.A., 2010). These not only include the symptoms of the three groups, but they also incorporate secondary symptoms like weight loss and autonomic and sleeping disturbances. Additionally, it's a disease that is relatively easy to target when compared to others because it's caused by a mutation (CAG repeat elongation) present only in one copy of the HTT gene, while other genetic diseases usually affect more than one gene. The articles studied in the theoretical framework also mentioned that the disease is caused by a specific sequence in the mHTT gene that is caused by the elongation of the CAG trinucleotide (Alkanli et al., 2022), (R.A., 2010). For this reason, there's a bright future in developing new treatment options for HD that will hopefully be more effective in regulating the symptoms and thus improving the quality of life of the patients.

CRISPR-Cas9: A Powerful Gene-Editing Technology

The interviewees who responded to the CRISPR-Cas9 questions agreed that CRISPR is a very efficient technique to modify the genome, that it's more advanced and easy to use than previous technologies, and that it has a bright future. CRISPR-Cas9 is known for being a very specific technique that can target genes through the inducement of a complex composed of the sgRNA, which is designed to match the sequence, and Cas9, the protein that cuts the sequence. The formation of the sgRNA-Cas9 complex and the mechanisms by which it edits DNA—that is, by detecting the PAM sequence, binding to the complementary DNA sequence, and inducing DSBs—were also mentioned in the theoretical framework (Alkanli et al., 2022). Right now, CRISPR-Cas9 is mainly applied to plants to improve crop fertility, animals (in livestock), and research for its use to treat genetic diseases. Investigation is done in cultured cells, mouse models, bacteria, and other microorganisms.

While CRISPR-Cas9 is a biotechnological technique with a lot of potential, it has several challenges that must be addressed before its implementation on a larger scale. CRISPR-Cas9 modifies DNA, which is a complex molecule that humans don't know a lot about; therefore, CRISPR-Cas9 could have unexpected effects on DNA that could hurt the organism. Dr. Jhon Fredy Betancur and Dr. Natalia Garcia highlighted the possibility of the complex binding to a homologous sequence to the one being targeted, which could cause side effects and alter cellular functions. While the risks of the process of the CRISPR-Cas9 complex binding to the DNA sequence were not mentioned in the theoretical framework, the risks of the repair mechanisms like Homology Directed Repair (HDR) and Non-Homologous End Joining (NHEJ) were mentioned. It was found that NHEJ is more prone to error than HDR because it has a high probability of INDEL mutations that can impact the organism negatively (Alkanli et al., 2022).

Another issue with CRISPR-Cas9 is that it's easier to use it on microorganisms, plants, or animals, but using it in humans brings many ethical concerns. For this reason, as the interviewees said, many CRISPR-Cas9 experiments in humans are probably not published.

One of the main fields of action for CRISPR-Cas9 is medicine, specifically for genetic diseases. While Dr. Jhon Fredy Betancur believes that CRISPR-Cas9 will have the capacity to cure genetic diseases in the future, Dr. Natalia Garcia believes that it will be a treatment, not a cure. This shows that the future of CRISPR is still undefined and that it requires further experimentation to prove its utility. Additionally, CRISPR will be a very expensive treatment because of all of the research that it requires and its mechanization is expensive. Also, transitioning from an animal model to a human will be expensive and complex. This group of interviewees, however, didn't mention the possible combination of CRISPR-Cas9 and regenerative medicine, through iPSCs, which was highlighted in the theoretical framework. The use of CRISPR-Cas9 in medicine can be accomplished by reprogramming patient-derived somatic cells, editing them with CRISPR-Cas9, and then differentiating them into the necessary cell type (Alkanli et al., 2022).

Scientists defend that the results of CRISPR-Cas9 research are promising. It is a technology that has a lot of potential due to its mechanisms, but it still requires more research and future experiments in humans for it to be trusted on a larger scale. It's important to note that in many countries, like Colombia, there are currently more facilities for working with CRISPR in microorganisms and plants than in animals or humans. The technique has a bright future, but several accommodations must be made to how it's delivered, and hopefully, new discoveries will continue proving its utility.

CRISPR-Cas9 as a Promising Future for HD's Treatment or Cure

The answers to the questions about CRISPR-Cas9 as a treatment for HD were analyzed. The answers of Dr. Diana Castellanos, Dr. Kyle Fink, and Dr. Natalia Garcia were compared and contrasted. With this analysis, a general conclusion of the topic was obtained.

Interview Questions Analysis

Question 1: Do you believe that CRISPR-Cas9 is a viable option to treat HD?

The three interviewees agreed in the fact that CRISPR-Cas9 is a viable option to treat HD because it is not as complex as other techniques that were used previously, it can target a specific gene, and HD is a great model for CRISPR-Cas9 testing because it's only caused by one gene, which is the mHTT gene. The experiments that were studied in the theoretical framework, especially those with mouse models and patient-derived cell lines, were successful in targeting the HTT gene (Ekman et al., 2019), (Kolli et al., 2017), (Wan et al. , 2016), which proves that CRISPR-Cas9 is a viable option to treat HD. However, the three researchers also highlighted that CRISPR-Cas9 has many risks and other considerations that must be taken into account before it's used to treat HD. They underscored that it's very hard to control CRISPR-Cas9 because it can affect unintended parts of the genome and cause side effects; in the process, it could cause other diseases, such as cancer. The risk of cancer that this treatment presents wasn't found in the theoretical framework section, and it's uncertain if it was an irrelevant point in the articles that discussed the topic or if the research projects that were studied haven't found cancer as a risk. Also, CRISPR-Cas9 can be a viable treatment according to the stage of the disease in which it's implemented: it will usually be more effective if it's done right after the disease is diagnosed and after the onset of the disease. This is more like a hypothesis than an idea that has been scientifically proven since there still haven't been any experiments in HD patients; since the

theoretical framework analyzed *in vivo* and *in vitro* studies, this point of view wasn't mentioned. Finally, the interviewees said that it's difficult to reach all of the nervous cells that are affected, also referred to as the proliferation or reproducibility of the method. It would require a more mechanized process. For this reason, more research is needed, but CRISPR-Cas9 has great potential as a treatment for HD.

Question 2: Do you believe that CRISPR-Cas9 could regulate the symptoms of HD, such as chorea?

The three interviewees agreed that CRISPR-Cas9 has the potential to regulate the symptoms of HD because that is one of the main purposes of the treatment. Since the disease is caused by a mutation in one gene, then cutting this gene will probably help in reducing the effects of the mutated gene, including neurotoxicity, cognitive impairments, emotional distress, and chorea. In the theoretical framework, an experiment with R6/2 mice was studied, and the Cas9 activity in their genomes was successful in decreasing hindlimb clasping at most weeks (Ekman et al., 2019), which shows the capacity of CRISPR-Cas9 to regulate motor dysfunction. In the same study, the mice injected with SaCas9 had 40% fewer mHTT levels than those that weren't injected with SaCas9. There was another study that used CRISPR-Cas9 to disrupt the region that regulates mHTT expression (uORF) in YAC128 HD mice (Kolli et al., 2017), which proves, as Dr. Kyle Fink said, that HD can also be treated by targeting the regions involved in the gene expression of HTT. The other study that was analyzed in the theoretical framework, which used different sgRNAs to excise the PAM sequence and the mHTT gene on HD-patient-derived neural precursor cells (NPCs) and iPS cell lines, proves again that CRISPR-Cas9 is a great option to treat HD.

However, the three interviews also said that the regulation of the symptoms depends on the time of the disease in which CRISPR-Cas9 is applied. If it's done years before the onset of the symptoms (in prediagnosed patients), then it'll be more easy to prevent the development of symptoms in the future. Nevertheless, interventions that are done after the progression of the disease will present more challenges in controlling the symptoms. The specialists also agreed that the treatment is intended to regulate all the symptoms of the disease as a whole, not specific symptoms like chorea, but that the symptoms that are regulated might vary among patients.

Question 3: Do you believe that CRISPR-Cas9 can be a definitive cure for HD in the future?

The answer to this question wasn't the same for the three interviewees. Dr. Kyle Fink was the most faithful because he believes that CRISPR-Cas9 can cure the disease, taking into account that the technology cuts the gene that causes HD. He thinks that the capacity of CRISPR-Cas9 to cure HD depends on the stage of the disease in which it's used; it will most probably be a cure if it's implemented very early. On the other hand, Dr. Diana Castellanos believes that CRISPR-Cas9 will probably be a treatment for the disease, rather than a cure and that if it were to be a cure then it would take a lot more time of research. Finally, Dr. Natalia Garcia thinks that the concept of cure is very broad and complex and that curing the disease would also require intervening in the process of evolution of humans since new mutations always appear and depleting CAG repeats completely is very difficult. These three different points of view prove that the concept of CRISPR-Cas9 as a cure for HD is ambiguous and still in the process of investigation: some believe it can be a cure, while some think it cannot. CRISPR-Cas9 is seen more as a possible treatment rather than a cure because there are still several complications or challenges in the technique that must be solved.

Question 4: Do you believe that it would be complex to implement CRISPR-Cas9 as a treatment for HD in medical centers around the world in terms of necessary materials and effectiveness?

The three interviewees agreed on the complexity of implementing CRISPR-Cas9 as a treatment for HD in terms of the availability of materials and the high costs that this treatment might have. Dr. Kyle Fink mentioned that the main challenge is that there's still no mechanized way of inducing CRISPR-Cas9 into the affected brain cells, making it difficult to deplete the mutation completely. Delivery is difficult because Cas9 is a very big protein. Dr. Diana Castellanos mentioned the complexity in terms of the high cost that CRISPR-Cas9 would have and the struggle there would be to take this technology from countries like the US or Europe to Latin America. This can be proven by the fact that most of the scientific papers researching this topic that were found and analyzed in the theoretical framework come from the United States, Europe, and countries in Asia like Turkey and South Korea. Finally, Dr. Natalia Garcia said that it would be difficult to implement it because there's still a lot of research missing; scientists are still in the phase of multicentric studies (like the studies from the theoretical framework) and there are many considerations that must be addressed, such as the danger of cancer development. The complexities of CRISPR-Cas9 treatment for HD are mainly based on costs, the unavailable mechanization of the technology, and the risks that it poses.

Question 5: Do you believe that it would be complex to implement this treatment in Colombia/US? (Depends on the nationality of the interviewee).

The three interviewees agreed on the complexity of implementing this treatment either in Colombia or the US (depending on the nationality of the interviewee). They all mentioned that the main factor that complicates it is the cost; in any location of the world, CRISPR would be

very expensive and difficult to afford for both the medical centers and the HD patients. Dr. Kyle Fink mentioned that these high costs are due to the amount of money and time that scientists invest in research and that a CRISPR-Cas9 treatment would cost around 4 million dollars. On the other hand, Dr. Diana Castellanos highlighted that Colombia can't pay for this treatment, especially because the EPS limits decisions in Colombia's medical system, and it will be very hard to bring this technology from other countries to Colombia. Similarly, Dr. Natalia Garcia mentioned that it would be complex to bring this technology to Colombia because of the costs and materials, and also because research on CRISPR-Cas9 as a treatment for HD is still limited.

Question 6: Are there significant risks for HD patients when treating them with CRISPR-Cas9?

The three interviewees underscored that there are notable risks to the patient's health when implementing CRISPR-Cas9 as a treatment for HD. It can be dangerous because, even though CRISPR-Cas9 is highly specific, there's still an error margin that can lead it to affect surrounding genes, cause mutations and target unintended sequences of the genome that contain CAG repeats, and all of this can end up in the development of side effects or other diseases, including cancer. Dr. Kyle Fink mentioned the targeting of the mHTT gene and the Double Strand Breaks (DSBs) induced by Cas9 can cause effects in the genome like indels, base-pair alterations, and chromosomic distribution effects like translocation and duplication. The risk of cancer mainly takes place when scientists try to repress the tumor protein P53 because its absence enables a higher rate of CRISPR-Cas9 activity; P53 is essential to prevent cancer development, so its absence, while enabling CRISPR, can cause tumors. Surprisingly, the targeting of the P53 tumor protein wasn't found in the papers analyzed in the theoretical framework. On the other hand, Dr. Diana Castellanos explained that CRISPR-Cas9 could

probably destabilize the genome of the host, and cause conditions like arrhythmia, cancer, and other side effects. However, these are just possibilities, and it all depends on the patient, so many applications to HD patients would have to be done to determine this. Both Dr. Kyle Fink and Dr. Diana Castellanos agreed that, regardless of the risks, HD is a very severe disease that has devastating effects on its patients, so it's better for HD patients to attempt the treatment and assume the side effects than having to endure the disease. Finally, Dr. Natalia Garcia also mentioned the risks of this treatment in terms of the alterations that it might have in unintended parts of the genome, causing diseases like cancer or other side effects. These responses show that this treatment's risks are based on the molecular effects that it can have, in terms of altering the genome of the host in other regions in an uncontrolled manner, and how the main disease that can be caused after this is cancer, due to the possibility of affecting genes or proteins that regulate mitosis.

Question 7: Have there been experimentations with CRISPR-Cas9 *in vivo* or *in vitro* in Colombia/US up until today? (Depends on the nationality of the interviewee).

The three interviewees were sure that experimentations *in vivo* and *in vitro*, especially in mouse models and cultured cells, respectively, have been done in the United States. However, Dr. Diana Castellanos and Dr. Natalia Garcia aren't sure of this research inside Colombia. They don't know about companies or projects that are working on this; therefore, they believe that these experimentations are being done in other countries, like the US, and will later be brought to Colombia. This shows that CRISPR-Cas9 is an advanced technology that not all countries have the same access to, making it complex to take it around the world. Again, this is proven by the fact that the *in vivo* and *in vitro* experiments studied during the theoretical framework were done

in countries far from Colombia. However, it's possible that studies in Colombia are still in progress and haven't been published.

Question 8: Has CRISPR-Cas9 already been implemented as a treatment for an HD patient? If yes, was it successful?

The three interviewees said that CRISPR-Cas9 still hasn't been implemented as a treatment for an HD patient in any part of the world. This can also be proved through the theoretical framework, since no experiments in human patients were found. Instead, the experiments found were done in cultured cell lines and mouse models. Nevertheless, they all believe that this will be accomplished in the future, even though a lot more research must be done before this.

Question 9: Has CRISPR-Cas9 been implemented as a treatment for an HD patient in Colombia/US? (Depends on the nationality of the interviewee).

The three interviewees agreed that CRISPR-Cas9 hasn't been used as a treatment for an HD patient either in the US or in Colombia, taking into account that it hasn't been implemented in any part of the world.

General Conclusion

CRISPR-Cas9 has a great potential as a treatment for HD; it can target the mHTT gene, which is composed of the expanded CAG repeat, and this sequence is responsible for all of the molecular mechanisms and symptoms that take place during HD. The mHTT gene is responsible for coding for the glutamine protein aggregates that cause neurotoxicity and mitochondrial dysfunction, and this generates the three groups of symptoms of HD: cognitive, emotional and motor. Since the mHTT gene is the source of the disease, and it can be removed completely

through DSBs induced by the CRISPR-Cas9 complex, then the technology is a great option to treat or cure the disease.

CRISPR-Cas9 is a highly specific technology that has been worked on throughout the last few years. Specific research on CRISPR-Cas9 for HD has been done through the use of *in vivo* models, usually mice, and *in vitro* models like iPSCs that have been obtained from HD patients. These have been successful, showing a slower disease progression in mouse models, for example. This is the current stage of research on CRISPR-Cas9 as a treatment for HD; it still hasn't been applied to an HD patient. Though this might be soon, most specialists believe it will still take time.

The CRISPR-Cas9 technology, while posing advantages, also presents challenges that limit its capacity to work as a treatment or cure for HD. The CRISPR-Cas9 complex might bind to a gene that is homologous to the one that was targeted through sgRNA, and this can cause mutations, cancer, or other side effects. The genome is very wide and complex, so scientists must work on it with a lot of care. Additionally, approaching the brain is very difficult, and this makes it necessary to find a mechanized method with which CRISPR-Cas9 can be delivered to the affected neurons in HD. While delivery or injection methods for CRISPR-Cas9 already exist, they're not efficient enough in reaching the necessary areas, and that would make the treatment process ineffective.

It's important to note that, while CRISPR-Cas9 poses significant risks for the patient, HD is the perfect disease model to continue refining this treatment option. HD is caused only by one gene, which can be easily targeted and cut by the CRISPR-Cas9 complex. Additionally, HD is a devastating disease that has a long-lasting impact on both the patient and the family, and there's still no cure or completely useful medications for it, causing the patient's quality of life to drop

significantly. Due to the conditions of HD, most of its patients would probably attempt CRISPR-Cas9 and assume the risks; the quality of life of HD patients can be worse than the side effects caused by the technology. For this reason, CRISPR-Cas9 may be a perfect fit for HD.

When the findings of the interviews were compared with the findings of the theoretical framework, there weren't very notable contradictions. There were small differences: for example, it was found in the theoretical framework that the first symptoms of HD are psychiatric and cognitive, while Dr. Diana Castellanos said in the interview that the first symptoms are motor. Additionally, there were important considerations in the implementation of CRISPR-Cas9 as a treatment for HD that weren't mentioned in the theoretical framework but were mentioned in the interviews, or vice versa. For example, P53 wasn't mentioned in the theoretical framework, and regenerative medicine was little mentioned in the interviews. Apart from this, most of the interview responses connected with the theoretical framework. It can be concluded from both that research on this topic is still in a stage of *in vitro* and *in vivo* studies, which have been successful in reducing mHTT expression and regulating motor symptoms in mice. These studies show the potential of the technology, but experiments in humans still haven't been reported, showing that scientists must continue research until they reach the next stage.

The general and specific objectives of the research project were accomplished. From the general objective, there was mainly an approach from the area of molecular biology and a study of both theoretical documents, which contained explanations of the molecular mechanisms underlying the treatment, and practical applications, focused on studying the research with CRISPR-Cas9 *in vivo* and *in vitro* and interviewing specialists. With this information, it was possible to determine if CRISPR-Cas9 is effective in treating HD. The first specific objective, which was to investigate the molecular mechanisms underlying HD, was accomplished by taking

scientific papers as a reference. The second specific objective was also addressed throughout the project since most of the studies explained *in vivo* and *in vitro* applications with CRISPR-Cas9. The third specific objective was achieved by comprehending that HD is a genetic disease, and that, for this reason, it is a perfect model to work on CRISPR-Cas9. Finally, the last specific objective was addressed throughout the last section of the project and analyzed with the use of both scientific papers and interviews with specialists mentioned previously.

The hypothesis established at the beginning of the project was correct. CRISPR-Cas9 is a technology that has the potential to treat or cure HD, but it still requires further investigation to be implemented like this. Scientists are still in the stage of research; they haven't moved on to experimenting with humans. The moment they have the chance to do this while assuming the risks, they'll move into the next stage of the treatment application and each time get closer to implementing it on a larger scale. The time it will take to start testing with HD patients is uncertain, but it is sure that many research projects are working on this, which is promising for the future use of the technology as a treatment for HD, thus improving the public health system and the quality of life of the patients.

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Annexes

Annex 1: Informed Consent

Informed Consent

Month, Day, 2025

Name of the research: "The Use of CRISPR-Cas9 Genetic Modification as a Treatment for Huntington's Disease."

Colegio Granadino.

Researcher: Isabela Osorio Arbeláez.

Advisor: Jillian Holmes.

SIP Director: Never Betancur Soto.

The fundamental idea of this research is focused on trying to demonstrate the effectiveness and feasibility of implementing CRISPR-Cas9 genetic modification as a treatment or possible cure for HD on a large scale and thus improve the quality of life of the patients.

The undersigned hereby state that we know the purpose of the research and authorize the use of a recorded interview and the information contained therein, framed within the regulations and ethics of that have to do with copyright.

_____	_____
Professional's Name	Isabela Osorio Arbeláez
Profession and Association's Name	Researcher

Annex 2: Email Requesting Interview Format

Interview Request - CRISPR-Cas9 as a Treatment for Huntington's Disease Senior Independent Project

Good afternoon **Professional's Name**, I hope this email finds you well.

My name is Isabela Osorio and I'm a twelfth grade student in Granadino, Villamaría, Caldas, Colombia. I'm currently doing a Senior Independent Project (SIP) entitled "The Use of CRISPR-Cas9 Genetic Modification as a Treatment for Huntington's Disease." I'm in the practical part of my research project, and I decided to interview specialists in my areas of interest.

I read about the research that **professional's institution** is currently conducting and I'm interested in interviewing you about Huntington's Disease and the use of CRISPR as a treatment for it. This would be a semi-structured interview, where I'd ask some questions about this topic. It can be either through text, where you would send me the answers to the questions, or through a video call, where you would answer the questions live.

Please let me know if you're available for the interview, and we can either schedule a meeting or I can send you the questions through email. I'd also send you an informed consent directly from the school so that you legally accept the interview request.

Thank you, I really appreciate your help, and I'm looking forward to your response,

Isabela Osorio

Annex 3: Interview Questions - Huntington's Disease

Question 1: How prevalent is HD, and to what extent is it considered common or rare?

Question 2: Does HD have a significant impact on the Colombian/US population? (Depends on the nationality of the interviewee).

Question 3: Do you believe that HD has a significant impact on the patient and his/her family?

Question 4: Which is the first symptom that usually presents in HD patients?

Question 5: Do all HD patients show similar symptoms and development of the disease, or does this vary?

Question 6: If this varies, what does it depend on?

Question 7: Which treatments are usually implemented for HD patients?

Question 8: Are these treatments useful in regulating the symptoms of HD?

Question 9: Has any definitive cure for HD been discovered, or is it in process?

Question 10: In case there's an HD cure in process, do you believe it will be successful?

Annex 4: Interview Questions - CRISPR-Cas9

Question 1: Do you believe that CRISPR-Cas9 is an efficient technology to modify the genome?

Question 2: Do you believe that CRISPR-Cas9 has a high error margin or is very risky?

Question 3: Has the CRISPR-Cas9 technology been implemented in the medical field up until today? If not, in which fields is CRISPR-Cas9 used during actuality?

Question 4: If CRISPR-Cas9 has been used in the medical field, do you believe it has worked or been used effectively? Why?

Question 5: Do you believe that CRISPR-Cas9 has the potential to cure genetic diseases? Why?

Question 6: Up until today, has CRISPR-Cas9 been used to modify a human genome with the purpose of treating a disease?

Question 7: Do you believe that the experiments that have been done with CRISPR-Cas9 have been successful?

Question 8: Do you believe that the amount of times that CRISPR-Cas9 has been experimented are enough to trust on implementing the technology in the medical field on a larger scale?

Question 9: Do you believe that the successful outcomes of experiments with CRISPR-Cas9 in cultured cells and mouse models support the fact that it could be successful in treating a human disease?

Question 10: Has CRISPR-Cas9 been used in Colombia/US? If yes, for what? Has it been used to treat diseases? (Depends on the nationality of the interviewee).

Question 11: Do you believe that CRISPR is a viable option to treat genetic diseases

in terms of its cost and necessary materials?

Annex 5: Interview Questions - CRISPR-Cas9 as a Treatment for Huntington's Disease

Question 1: Do you believe that CRISPR-Cas9 is a viable option to treat HD?

Question 2: Do you believe that CRISPR-Cas9 could regulate the symptoms of HD, such as chorea?

Question 3: Do you believe that CRISPR-Cas9 can be a definitive cure for HD in the future?

Question 4: Do you believe that it would be complex to implement CRISPR-Cas9 as a treatment for HD in medical centers around the world in terms of necessary materials and effectiveness?

Question 5: Do you believe that it would be complex to implement this treatment in Colombia/US? (Depends on the nationality of the interviewee).

Question 6: Are there significant risks for HD patients when treating them with CRISPR-Cas9?

Question 7: Have there been experimentations with CRISPR-Cas9 *in vivo* or *in vitro* in Colombia/US up until today? (Depends on the nationality of the interviewee).

Question 8: Has CRISPR-Cas9 already been implemented as a treatment for an HD patient? If yes, was it successful?

Question 9: Has CRISPR-Cas9 been implemented as a treatment for an HD patient in Colombia/US? (Depends on the nationality of the interviewee).